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Enclosed Aquatic Multispecies Test System for Testing Microbial Pest Control Agents with Non-Target Species

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AN ENCLOSED AQUATIC MULTISPECIES TEST SYSTEM FOR TESTING MICROBIAL PEST CONTROL AGENTS WITH NON-TARGET SPECIES

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ABSTRACT

An enclosed test system was developed in which multiple species of aquatic animals and plants were tested experimentally for adverse non-target effects of wild-type and genetically altered microbial pest control agents (MPCAs). The test system consisted of components that were inexpensive and readily available from aquaculture supply companies or from retail pet shops that carry tropical fish aquaria and supplies. A variety of marine and freshwater non-target animal and plant species (NTOs), representing diverse phylogenetic taxa and trophic levels, were collected from wild populations or purchased from commercial suppliers.

Four different types of model MPCAs were tested in the multispecies system. These included two different strains of the mosquito pathogen Bacillus sphaericus, a strain of Pseudomonas putida (used as a model for the genus), and the insect baculovirus AcMNPV. The fate, persistence, and infectivity of these model MPCAs were evaluated using traditional microbiological and histological methods, as well as specific microbiological assays for model MPCAs that were altered by addition of a unique genetic marker. For two of the model MPCAs, gene probes were used as a detection method to track the MPCA in the test system water and NTOs.

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INTRODUCTION

Microbial pest control agents (MPCAs), also known as biological control agents or "biorationals," are microbial agents intended for use in controlling detrimental insects, weeds, and other pests (Couch and Rao, 1983; Couch and Martin, 1984). Because they may be applied in rather large quantities (Mulligan et al., 1980) or repeatedly applied in smaller quantities to areas outside the normal geographic range of the wild type pathogen (Davidson et al., 1984), it is important that test data be obtained, prior to field application, which will help to predict the fate and persistence of MPCAs in the environment and their effect on non-target organisms that would be exposed to a given MPCA as a result of normal field application. Acquisition of such information becomes even more important when the application of genetically altered MPCAs is considered (Environmental Protection Agency, 1982).

Controlled tests are clearly needed in which wild-type and genetically altered MPCAs are tested in self-contained systems in the laboratory with organisms representing species which are not pests (non-target organisms, NTOs), but are likely to become exposed as a result of the field application of MPCAs. Data from these controlled laboratory studies (which approach, but cannot mimic, actual field conditions) may be used as guidelines for further studies, the goals of which will be used to assess the safety and/or hazards of proposed future uses of genetically altered MPCAs.

The purpose of the project reported here was to develop a functionally closed aquatic multispecies test system in which the study of MPCAs could be accomplished in a manner that models an ecosystem (Lundgren, 1985) and utilizes as many different, yet readily available types of NTOs as possible. Therefore, the two major objectives of the study were: 1) to develop a relatively simple, easily replicated, and inexpensive recirculating tank system in which multiple species of aquatic (estuarine, marine, or freshwater) animals and plants could be maintained in direct or indirect contact with each other for extended study periods with an introduced MPCA; and 2) to conduct studies on the fate and effects of model MPCAs on the non-target organisms in the test system.

MATERIALS, METHODS AND RESULTS

TEST SYSTEMS

Five different enclosed recirculating tank designs were constructed and tested as potential multispecies test systems for tests with model MPCAs (wild strain or genetically altered).

Test System No. 1

To provide a standard from which to compare various configurations of multispecies test tanks and aquaria, a 120 L glass aquarium was assembled to duplicate as closely as possible the principal design features of those described by Foss and Couch (undated, internal EPA report) and by Fournie et al. (1987 and 1988).

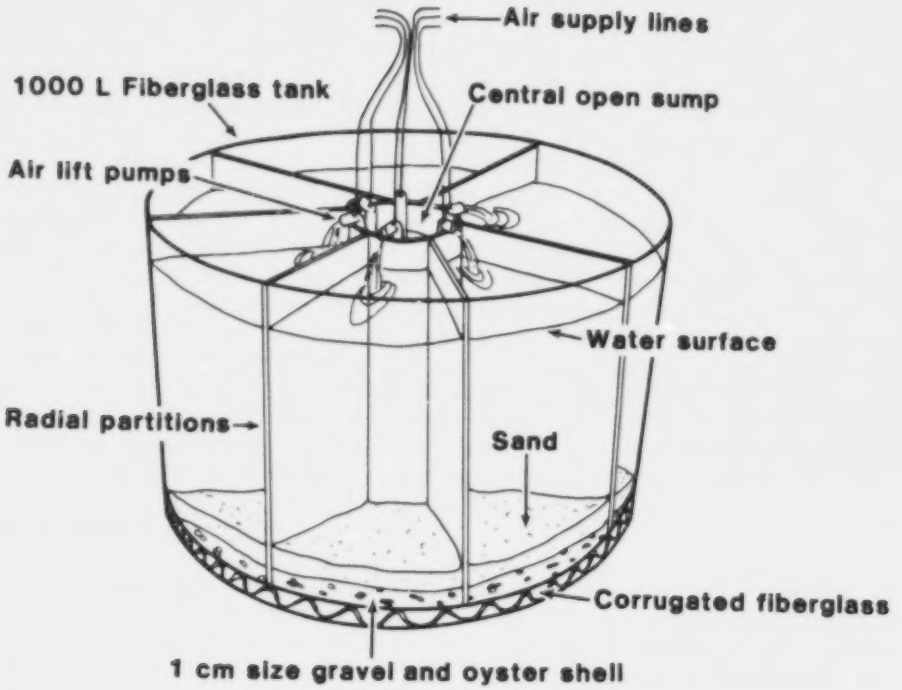
Test System No. 2

This experimental test system consisted of a 1,000 L round fiberglass tank equipped with its own undergravel biological filter, and six compartments separated by radial partitions constructed of flat fiberglass panels (Figure 1). Seawater within the tank could be recirculated through the undergravel filter through a central sump, or be moved from any compartment to another via six moveable airlift pumps. A number of the species in Tables 1 & 2 were maintained successfully in the system for more than 60 days, in numbers large enough to provide adequate sample sizes for microbiological and histological sampling in model MPCA tests. However, the system was found to have one significant deterrent to its practical use: the animals contained in it could not readily be inspected or even counted without literally draining the tank.

Test System No. 3

This test system was designed to incorporate the advantages of systems No. 1 and No. 2, while eliminating their major disadvantages. System No. 3 was constructed using a 400 L commercially available Plexiglass tank. Initially, it was equipped (Figure 2) with both undergravel and vertical biological filters consisting of crushed oyster shell. In actual use the undergravel filter was found to be unnecessary, and in subsequent tests with this tank, only the vertical biological filters were utilized. The vertical filters were held in place by fiberglass window screen mounted on rigid frames made from 6.35 mm thick PVC flat stock, and these assemblies were held in place in the tank by pairs of vertical PVC strips glued to the tank walls to form slots. The vertical filters had two functions: to provide biological filtration, and to physically separate species in the tank and thus prevent unplanned predation. Salicornia bigelovii (an estuarine vascular plant) seedlings planted in these dividers grew normally.

Airlift pumps and an externally affixed electric pump provided both aeration and circulation of water through the vertical biological filter matrices. The airlift pumps were moveable and provided direct mixing of adjacent sections of the tank. The system performed well and was easily managed; species reared within it could be easily observed, counted, and sampled; some test species actually reproduced in the system during the 60-day trial period.



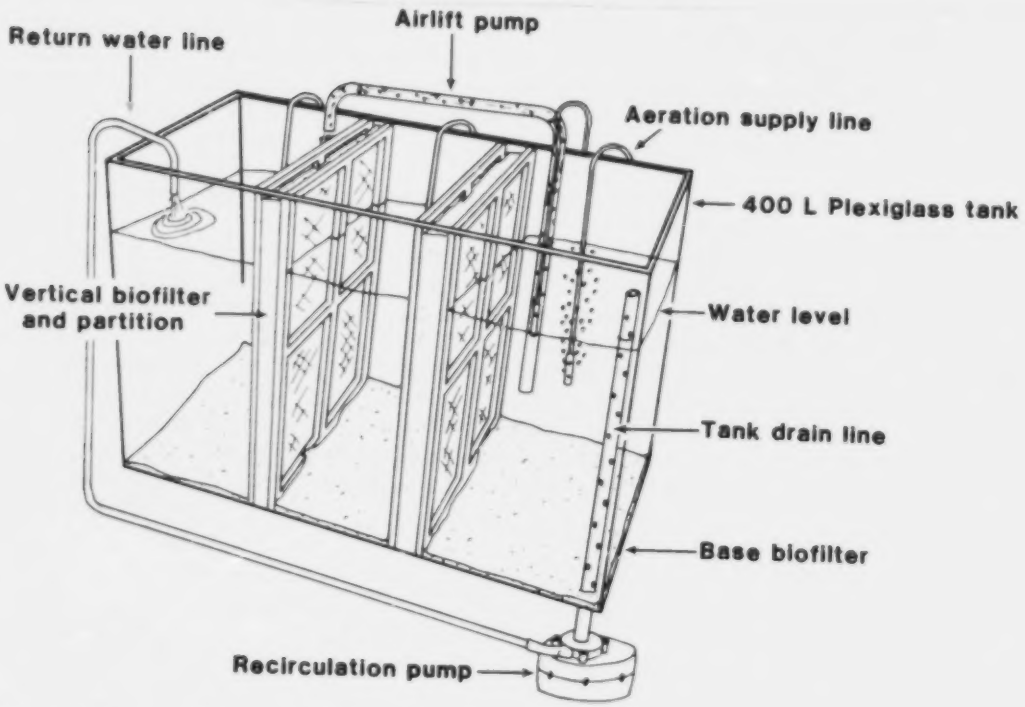


Figure 1. Schematic of the multispecies test system No. 2.
Figure 2. Schematic of the multispecies test system No. 3.

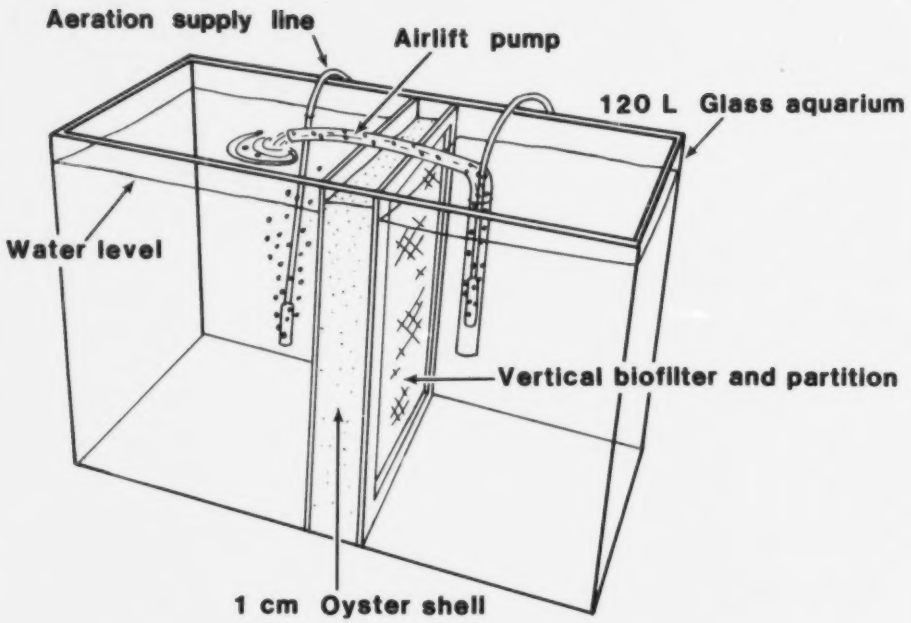
Test System No. 4

Despite the advantages of System No. 3, its relatively high cost limited its practical use. Therefore, Test System No. 4 was constructed to combine the low cost and component availability advantages of System No. 1 (the E.P.A. system) with the functional advantages of System No. 3. This tank system was constructed using a standard 120 L (30 gallon) glass aquarium. Parallel strips of 6.35 mm thick PVC flat stock were cemented to opposite sides of the aquarium using silicone cement (Dow-Corning^{R}). As in System No. 3, the filter matrix was crushed oyster shell held in place with fiber glass window screen mounted on a frame of PVC flat stock. One of the principal advantages of System No. 4 was its vertical biological filter which tended to remove introduced particulate material (like the model MPCAs or phytoplankton introduced as food for filter feeding mollusks) much more slowly than does a bottom undergravel filter. Another advantage the vertical filter provided was physical separation of potential predators from prey species, thereby eliminating the need for several separate containment structures.

Test System No. 5

This system was identical to System No. 4, except that its biological filter matrix consisted of a polyester fiber pad (purchased as bulk furnace air filter material) in place of the crushed oyster shell (Figures 3 & 4). The advantage of the polyester filter over crushed oyster shell was that the former could be easily removed, cleaned, and replaced without draining the tank. The pH of seawater in tanks with polyester pad filters was maintained by suspending 1 L plastic beakers with perforated bottoms and half filled with crushed oyster shell or dolomite under the outlet of an airlift pump (Figures 4 & 5).

A further slight modification was made to eliminate the unanticipated problem of the non-target test animals becoming trapped in the biological filter. A perforated plastic sheet (made from undergravel aquarium filter kits) was installed on both sides of the vertical polyester pad filter (Figure 5). This prevented the filter pad from sagging and thus prevented the experimental animals from becoming trapped between the sides of the tank and the biofilter, while still allowing free flow of tank water through the biofilter. Because of the functional simplicity and relatively low costs of the original and modified System No. 5 tank type, this system was used in all tests with model MPCAs.



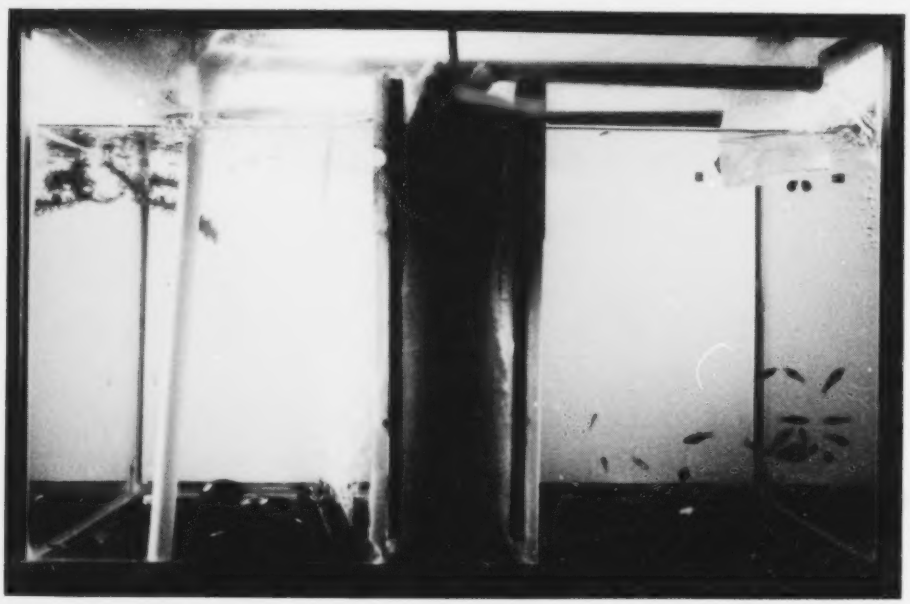


Figure 3. Schematic of the multispecies test system No. 4.
Figure 4. Photograph of the multispecies test system No. 5 as used in the saltwater MPCA Trials 1-5.

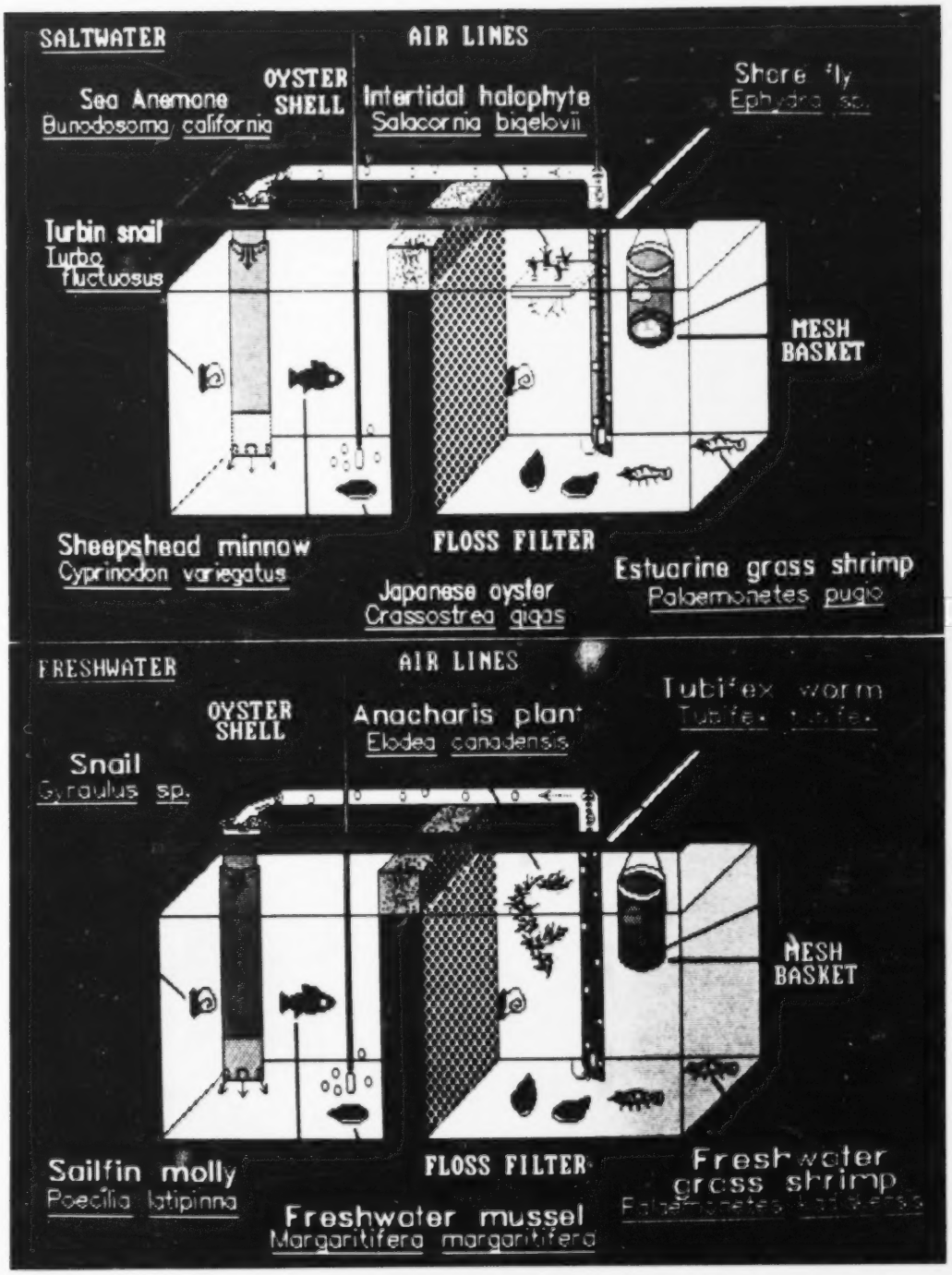


Figure 5. Schematics of the multispecies test system No. 5 as used in:
5a. the saltwater MPCA Trials 1-5.
5b. the freshwater MPCA Trials 6-7.

Table 1. List of marine species of indicator test organisms used in tests with model MPCAs, their original source, their culturability in the laboratory, and thier suitability as test organisms in tests with model MPCAs.

Marine Species	Source[1]	Lab Culture [2]	Test Organism Suitability
PLANT			
Salicornia bigelovii (intertidal halophyte)	Gulf of CA	RP	Poor
INVERTEBRATE ANIMALS			
Bunodosoma californica (sea anemone)	Gulf of CA	RP	Fair
Turbo fluctuosus (turbin snail)	Gulf of CA	CW	Excellent
Crassostrea gigas (Japanese oyster)	Gulf of CA	PS	Excellent
Palaemonetes pugio (estuarine grass shrimp)	Florida	RP	Excellent
Ephydra sp. (shore fly)	Gulf of CA	RP	Fair
VERTEBRATE ANIMAL			
Cyprinodon variegatus (sheepshead minnow)	Florida	RP	Good

[1] Source: Gulf of CA = collection sites near Puerto Penasco in Sonora, Mexico, on the Northern Gulf of Mexico.

[2] Lab culture: RP = reproducing laboratory colony established.
CW = captive wild colony successfully maintained in lab.
PS = experimental animals purchased from a commercial supplier and maintained in lab.

Table 2. List of freshwater species of indicator test organisms used in tests with model MPCAs, their original source, their culturability in the laboratory, and thier suitability as test organisms in tests with model MPCAs.

Freshwater Species	Source	Lab Culture[1]	Test Organism Suitability
PLANTS			
Elodea canadensis (Anacharis plant)	Commercial	PS	Excellent
INVERTEBRATE ANIMALS			
Tubifex tubifex (annelid worm)	Commercial	PS	Fair
Gyraulus sp. (snail)	Arizona pond	RP	Good
M. margaritifera (freshwater mussel)	Commercial	PS	Excellent
P. kadiakensis (freshwater grass shrimp)	Commercial	RP/PS	Good
VERTEBRATE ANIMALS			
Poecilia latipinna (sailfin molly)	Hawaii	RP	Excellent

[1] Lab culture:
RP = reproducing laboratory colony established.
PS = experimental animals purchased from a commercial supplier and maintained in the laboratory.

Table 3. List of plant and animal species acquired and tested in recirculating seawater and freshwater systems as candidate non-target species in multispecies tests with model MPCAs.

Species	Category	Habitat	Source
PLANTS			
Enteromorpha intestinalis	algae	marine	Gulf of CA
Salicornia bigelovii	vascular plant	"	" " "
Azolla caroliniana	water fern	fresh	pond, AZ
Limnobium spongia	duckweed	"	" "
Elodea canadensis	anacharis plant	"	commercial
INVERTEBRATES			
Bunodosoma californica	anemone	marine	Gulf of CA
Ceratonereis mirabilis	annelid worm	"	" " "
Sipunculus nudus	" "	"	" " "
Tubifex tubifex	" "	fresh	commercial
Gyraulus sp.	snail	"	pond, AZ
Margaritifera margaritifera	mussel	"	commercial
Turbo fluctuosus	snail	marine	Gulf of CA
Cerithidea mazatlanica	snail	"	" " "
Crassostrea gigas	oyster	"	" " "
Palaemonetes pugio	shrimp	marine	Florida
Palaemonetes kadiakensis	"	fresh	commercial
Penaeus stylirostris	"	marine	Gulf of CA
Mysidopsis bahia	"	"	Florida
Clibanarius digueti	hermit crab	"	Gulf of CA
Uca crenulata	fiddler crab	"	" " "
Ephydra sp.	shore fly	marine	Gulf of CA
VERTEBRATE ANIMALS			
Cyprinodon variegatus	minnow	marine	Florida
Poecilia latipinna	molly	fresh	Hawaii
Gambusia affinis	mosquito fish	"	commercial
Notropis lutrensis	red shiner	"	"

MPCA TESTS AND NON-TARGET TEST ORGANISMS

A variety of marine, estuarine and freshwater vertebrate and invertebrate animals and plants were acquired for potential use as non-target test organisms (NTOs) in model MPCA tests. The selection criteria for potential NTOs were that they should be common and readily available from cultured laboratory stocks, commercial suppliers, or from easily accessible wild populations. An effort was made in selecting plants and animals for use in the multispecies test system that represented diverse phyla and that, in the case of the animal species, represented different levels in the food web. The marine, estuarine, and freshwater species acquired and evaluated as candidate NTOs with model MPCAs are listed in Table 3. Those species found to be suitable for testing are listed in Tables 1 and 2.

MPCA Tests with Marine NTOs

The estuarine and marine NTOs used in MPCA Trials 1-5 were: the estuarine vascular plant, Salicornia bigelovii; the snail, Turbo fluctuosus; larvae of the shore fly, Ephydra sp.; the sea anemone, Bundosoma californica; the oyster, Crassostrea gigas; the grass shrimp, Palaemonetes pugio; and the sheepshead minnow, Cyprinodon variegatus (Table 1). Those species not readily available from commercial suppliers or from laboratory cultures were collected from wild populations along the Gulf of Mexico coast of Florida and from the Northern Gulf of California.

The saltwater plant (S. bigelovii) was supported in hydroponic culture on a styrofoam raft floated in the test tank seawater with its roots hanging free in the water. Juvenile sea anemones from their mass culture tank were allowed to grow on oyster shells, which were transferred into the test tanks. The shore fly larvae were confined to a net bag suspended in the test tank water.

MPCA Tests with Freshwater NTOs

The freshwater NTOs used in tests with model MPCAs included the freshwater anacharis plant Elodea canadensis, the annelid worm Tubifex tubifex, the snail Gyraulus sp., the freshwater mussel Margaritifera margaritifera, the grass shrimp Palaemonetes kadiakensis, and the molly (a finfish) Poecillia latipinna. All of the freshwater NTO species were acquired from commercial sources (Table 2), with the exception of the snail (Gyraulus sp.) and the molly (P. latipinna). The snail was collected from a fish culture pond in Southern Arizona, and the molly was originally collected from estuarine drainage ditches on the Island of Oahu, Hawaii.

Mass Culture of NTO Test Species

Culture and holding facilities for laboratory colonies of the marine and freshwater NTO species consisted of four totally self contained, recirculating multiple tank systems located in a

temporary sheet metal building and a plastic covered greenhouse on the grounds of the University of Arizona's Environmental Research Laboratory (Figure 6).

Specifically, each culture system consisted of three to four 1,500 L cylindrical fiberglass tanks that were plumbed with center drains. Each tank's center drain was fitted with a screened vertical stand pipe to regulate water depth and to prevent escape of the cultured species. Drain lines from tank sets were connected to large diameter PVC pipes through which water flowed by gravity to 4,000 L concrete water storage sumps (two below ground level septic tanks located just outside the metal building). Water from the sumps was pumped to 1,000 L biological filters with 1 hp swimming pool-type pumps, and returned through supply headers to the culture tanks using 0.5 hp submersible pumps located in the end of the each of the biological filter boxes opposite the water inlet (Figures 6-8).

The biological filters were fabricated "in-house" using plywood construction. Filter boxes were 4 ft by 8 ft by 2 ft deep. All surfaces were sealed with fiberglass resin and epoxy paint. Biological filter material consisted of polyester fiber pads (bulk furnace filter material) held in five vertical rows perpendicular to the axis of the filter box with heavy plastic net material in rigid PVC plastic frames. These filter pad units were held in place in the filter box in flanged slots fastened to the box sides. Receiving the incoming water from the storage sump was a 120 L polyethylene container (a trash can) filled with a mixture of crushed oyster shell, dolomite, and removable filter bags of activated charcoal. The oyster shell and dolomite functioned to regulate pH and alkalinity, while the charcoal was used to remove dissolved organic pigments and other dissolved organic materials from the system.

Feeds and Feeding Methods for NTOs

Marine and freshwater NTOs in mass rearing tanks and in the MPCA test tanks were fed once per day with live food organisms, frozen, or artificial feeds that were consistent with their feeding behavior and known nutritional requirements (Table 4). Thus, filter-feeding mollusks were fed cultures of planktonic algae once per day Walne, 1974), finfish and grass shrimp received chopped frozen squid, artemia nauplii and a commercial flake food daily, etc. NTO species that were not fed directly included the aquatic plants (Salicornia and Elodea), the snails, the tubifex worms, and the shore fly larvae. Except for Salicornia, all experimental NTOs appeared to do well (in terms of feeding activity, survival, general appearance, and histological condition) in test tanks and in mass culture tanks. Apparently, the cultural requirements for Salicornia were not met with the hydroponic culture method employed for culturing this plant in the MPCA test tanks. The plant did very well in those culture systems (Test Tank Systems No. 3 and No. 4) where it was cultured rooted in sand or oyster shell biological filter substrates, but it did poorly in hydroponic culture.

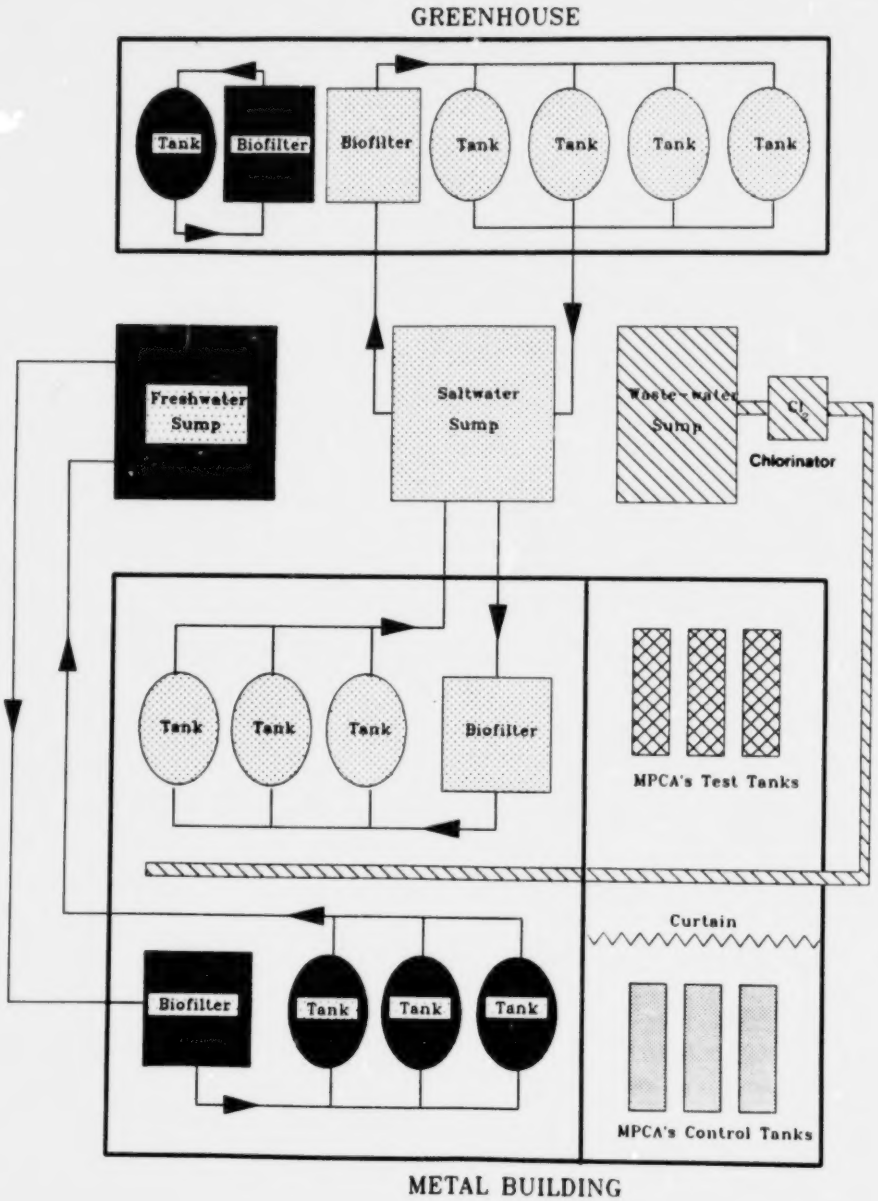


Figure 6. Schematic of the functional arrangement of culture and MPCA test systems located within the structures at the University of Arizona's ERL.

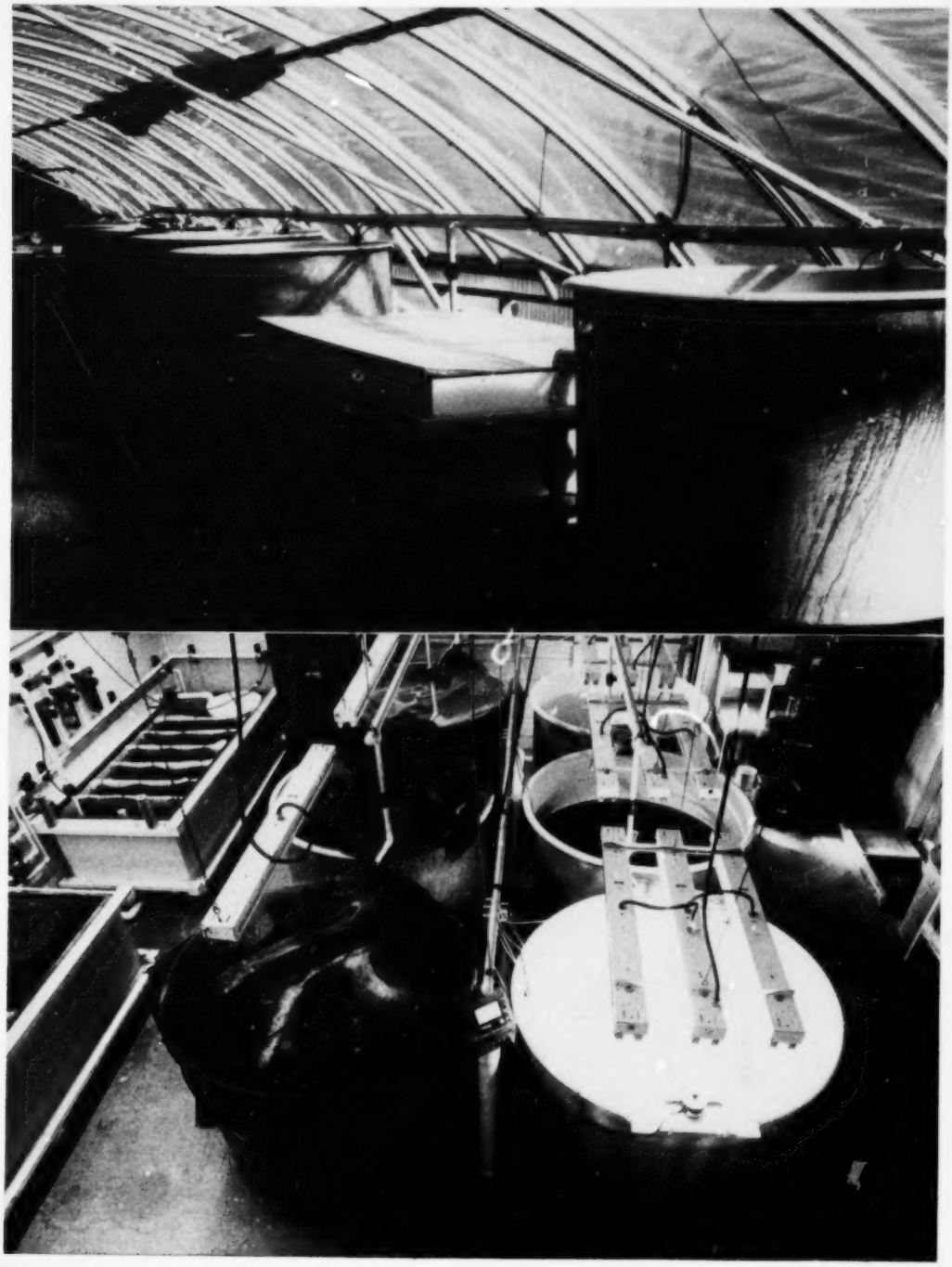


Figure 7. Photograph of the culture area in the greenhouse.
Figure 8. Photograph of the culture area in the metal building. The biological filter (far left) supports the round culture tanks used to rear saltwater NTO species.

Table 4. Feeds and feeding methods for non-target test animals.

NTO Species[1]	Food(s)	Feeding Frequency
Sea anemone (M)	artemia nauplii	daily
Turbin snail (M)	natural epiphytes, detritus	-
Japanese oyster (M)	phytoplankton mixture[2]: Isochrysis sp., Tetraselmis sp., and Chaetoceros sp.	daily
Grass shrimp (M)	Basic Food[3], chopped squid and artemia nauplii	daily
Shore fly larvae (M)	natural microflora	-
Sheepshead minnow (M)	Basic Food, chopped squid and artemia nauplii	daily
Tubifex worm (FW)	natural microflora	-
Snail (FW)	natural epiphytes, detrtius	-
Freshwater Mussel	Chlorella pyrenoidosa	daily
Grass Shrimp (FW)	Basic Food, chopped squid and artemia nauplii	daily
Sailfin Molly (FW)	Basic Food, chopped squid and artemia nauplii	daily

1 M = a marine species
FW = a freshwater species

2 Marine phytoplanktonic species were fed in approximately equal amounts daily. These were grown separately in mass unialgal culture using standard algae culture methods as found in manuals for mollusk culture.

3 Fish Basic Food, Worldwide Aquatics, Gardenia, CA

MODEL MPCAs TESTED

Four "model" MPCAs were tested with marine and freshwater NTOs in our test system. Used in these studies were three bacterial MPCAs (a spore forming Bacillus, a vegetative form of Bacillus, and a Pseudomonas), and a nuclear polyhedrosis virus (Tables 5 and 6).

Spore-Forming Bacillus sphaericus

Spores of Bacillus sphaericus (modified strain 2362; provided by Dr. A. Yousten, Virginia Polytechnic Institute and State University, VA and Dr. William F. Burke, Arizona State University, Tempe, AZ) containing the plasmid pLT103 (which encodes for neomycin resistance) were used in Trials 1 and 5. Many strains of B. sphaericus are known to possess insecticidal activity against mosquitoes (Brownbridge and Margalit, 1987; Davidson, 1981; Davidson et al., 1984). This strain of B. sphaericus is being developed commercially for use as a mosquito larvacide (Yousten, pers. comm.). The bacillus was also naturally resistant to streptomycin, and media supplemented with neomycin and streptomycin provided an excellent method to selectively isolate and culture the microorganism. Tryptose blood agar base (TBAB, Difco Laboratories, Detroit, MI), supplemented with 5 ug/ml neomycin (neomycin sulfate, Sigma Chem. Co., St. Louis, MO) and 100 ug/ml streptomycin (streptomycin sulfate, Sigma Chem. Co., St. Louis, MO), was used to produce the cultures of the bacillus from which spores were harvested.

Vegetative Cells of Bacillus sphaericus

Vegetative cells of B. sphaericus (modified strain 1593, thymine deficient; provided by Lisa D. Taylor and Dr. William F. Burke, Arizona State University, Tempe, AZ) harboring the plasmid pLT117 were used in Trial 2. The plasmid (pLT117), a ligation product of pTG402 and pUB110, encodes for neomycin resistance, and it contains the xylE gene. The xylE gene (Zukowski et al., 1983) expresses catechol 2,3-dioxygenase which converts catechol (0.5M, Sigma Chem. Co., St. Louis, MO) from colorless to a yellow product (2-hydroxymuconic semialdehyde) within a few minutes when sprayed onto growing colonies. Culture methods for this organism consisted of supplementing TBAB agar plates with Neomycin (at 5 ug/ml and thymine at 50 ug/ml). After overnight incubation, plates were sprayed with catechol solution and observed for yellow colonies. Because this Bacillus strain was a poor spore former, no effort was made to produce spores.

Table 5. List of model microbial pest control agents (MPCAs) used in this study.

MPCA	Plasmid in MPCA	Methods of Detection	Plasmid for Gene Probe
Bacillus sphaericus - spores - strain 2362 - streptomycin ^{R}	pLT103	Culture on TBAB plates with: - neomycin 5ug/ml - strep 100ug/ml	n/a
Bacillus sphaericus - veg. cells - strain 1593	pLT117 - Neo ^{R} - Xyl E gene	Culture on TBAB plates with: - neomycin 5ug/ml - thymine 50 ug/ml Yellow w/catechol	n/a
Pseudomonas putida - PPO200 - NX ^{R}	pEPA74 - Kana ^{R} - 400 bp insert of plant DNA	Culture on Pseudo F plates with: - Nx 500ug/ml -Kana 150ug/ml Gene probe	pEPA90 - in E. coli Ac80 - 400 bp plant DNA
AcMNPV (baculovirus) - occlusion bodies	n/a	Gene probe	- in E. coli JM83 JM83 (a pUC18 plasmid with a 1000 bp insert from AcMNPV)

Abbreviations used in Table:

Strep = streptomycin
^{R} = resistant
Kana = Kanamycin
Nx = nalidixic acid
TBAB = tryptose blood agar base
LB = Luria-Bertani culture media (Maniatis et al., 1982).

Table 6. List of MPCA trials run with marine and freshwater non-target organisms.

Trial Number	Test System	MPCA Tested (form used)	Principal Detection Method(s)
1	Marine	B. sphaericus 2362 (spores)	Unique genetic markers: (resistance to neomycin and streptomycin) Spore formation
2	Marine	B. sphaericus 1593; (vegetative cells)	Unique genetic marker: (microbiological assay for catechol 2,3-dioxygenase)
3	Marine	AcMNPV baculovirus (polyhedra)	Gene probe to the gene for polyhedrin
4	Marine	P. putida PPO200 + pEPA74 + Nx^R	Unique genetic markers: (resistance to nalidixic acid & kanamycin) Gene probe
5	Marine	B. sphaericus 2362 (spores)	Unique genetic marker: (resistance to neomycin and streptomycin) Spore formation
6	Freshwater	P. putida PPO200 + pEPA74 + Nx^R	Unique genetic markers: (resistance to nalidixic acid & Kanamycin) Gene probe
7	Freshwater	AcMNPV baculovirus (polyhedra)	Gene probe to the gene for polyhedrin

Pseudomonas putida with Genetic Markers

For Trials 4 and 6 vegetative cells of a genetically altered strain of Pseudomonas putida were provided by Dr. Fred Genthner, U.S. E.P.A., Gulf Breeze, FL. While this species of Pseudomonas has no uses presently as an MPCA, it was selected for use as a model MPCA for other members of the genus which are being developed for such use. This strain had been modified from the parent strain PPO200, by transformation with the plasmid pEPA74. The plasmid was constructed by inserting the pUC19 multiple linker sequence and a piece of plant DNA (approximately 400bp) into a plasmid pKT230 which contains Kanamycin resistance. The sequence of plant DNA was inserted between an EcoR1 site and a Pst1 site. This resulting pseudomonad was mutated in two separate genes on the chromosome to produce a strain resistant to high levels of nalidixic acid. The first mutation was made in a permease gene and the second in a DNA gyrase gene. The resulting strain is called PPO 220 + pEPA74 + Nx^R. In addition, the 400 bp of plant DNA was also inserted into a Pst1/EcoR1 site on a pUC 18 plasmid and then transformed into E. coli Ac80. This organism provided plasmid DNA for labeling, which was used as a gene probe. The genetic manipulations of the bacteria were performed by Dr. Genthner et al. at EPA Gulf Breeze Laboratory.

Aliquots of the bacteria that had been received from Dr. Genthner were thawed and plated on Pseudomonas F Agar (PsF) supplemented with 500 ug/ml nalidixic acid and 150 ug/ml Kanamycin. The high concentration of divalent cations in PsF enhances the productions of fluorescein, a yellow pigment. By using PsF agar, which is a differential medium for fluorescent pseudomonads, plus a high concentration of nalidixic acid and Kanamycin in the medium, we were able to selectively isolate and culture the genetically altered strain of P. putida from the MPCA test system and NTOs.

The Nuclear Polyhedrosis Virus AcMNPV

The nuclear polyhedrosis baculovirus (AcMNPV) from the lepidopteran Autographa californica was used as the model MPCA in Trials 3 and 7. Dr. Max Summers (Texas A&M University, College Station Texas) and Dr. Pat Vail (U.S.D.A., Riverside, CA) provided the strain of AcMNPV used (Summers and Smith, 1987). In addition, Dr. Summers provided the JM83 strain of E. coli that harbors a pUC18 plasmid, which contains a pAC HindV insert (1000 bp) of the central region of the polyhedrin gene of AcMNPV (Norander et al., 1983).

Experimental quantities of AcMNPV were produced from laboratory-reared colonies of the cabbage looper moth Trichoplusia ni; the original moths were provided by Dr. Tom Henneberry (U.S.D.A. Western Cotton Research Laboratory, Phoenix, AZ). Stock colonies of the moth were cultured under ambient laboratory conditions as follows: Adult cabbage loopers were held in 4 L glass jars with gauze tops until mating and egg laying had occurred. Larvae that emerged from the egg deposits were

collected and transferred to trays of an autoclaved lima bean diet (modified from Patana, 1969) supplemented with a mold inhibitor (0.3% propionic acid), and covered with a plastic film to prevent dehydration. After completion of the larval stages, pupae were collected from the trays and transferred to the 4 L jars to complete a colony culture cycle.

Larvae used to produce AcMNPV virus polyhedra were infected with the virus by addition of AcMNPV polyhedra to the lima bean culture media on day 6 (from egg). Day 15 larvae showing gross signs of heavy infection were collected. AcMNPV polyhedra were purified by homogenization, filtration, and centrifugation (Shapiro, 1981). Purified polyhedra were stored at 4 ^{o}C or at -80 ^{o}C until used in subsequent MPCA trials.

CONTAINMENT OF MPCAs IN THE TEST SYSTEMS

Figure 6 shows a schematic of the metal building in which the studies with model genetically altered MPCAs were conducted. The building is located at the extreme eastern end of the Environmental Research Laboratory grounds and is isolated from other experimental buildings by at least 30 meters (approximately 100 feet). The building was modified to provide a limited access "containment" area. A 2.2 m (~8 ft) high wall separated the experimental half of the building, where MPCAs were tested with NTOs in glass aquaria, from the entry portion of the building which also housed two of the four recirculating culture tanks systems used to culture the laboratory colonies of NTOs. Access to the experimental side of the building required use of rubber boots disinfected in a 200 ppm chlorine foot bath (prepared fresh every seven days from household bleach solution).

In each MPCA trial six multispecies aquaria were set up in the experimental half of the building (three test, three control). It was found that aerosol cross-contamination of the model MPCA (by Bacillus sphaericus spores) from exposed to control tanks took place in Trial 1. Cross-contamination was eliminated in subsequent trials by separating test and control tanks by at least 3 m (~ 10 ft), by installation of covers on the top of the tanks to contain aerosols, and by separating exposed and control tanks from each other by a plastic curtain room divider.

Nets used to remove NTOs from the tanks for sampling purposes were labeled and dedicated to a particular tank (to reduce cross-contamination) and disinfected separately in 100 ppm iodine (polyvinyl providine iodine; Fritz Egg Disinfectant, Fritz Chem. Co., Dallas, TX). The floor was mopped with 100 ppm pvp iodine periodically to further reduce the risk of cross-contamination. A common 5000 liter concrete sump (a modified septic tank) received waste water from both halves of the building. Water contained in the sump was continuously chlorinated (to > 20 ppm chlorine) using a floating swimming pool chlorinator and chlorine tablets, prior to periodic disposal when the sump had been filled to capacity.

GENERAL METHODS FOR MPCA TESTS

For all trials with model MPCAs, six of the Test System Type 5 (self-contained 120 L glass tanks: three test and three control) were used. Each of three replicate test and control tanks were stocked with 15 NTOs of each animal species. This provided a total of 45 animal NTOs of each species in test and control treatments of each MPCA test. This number was sufficient to provide for microbiological and histological sampling, as well as to allow for some loss due to possible natural and/or treatment related mortalities, while still providing for statistical confidence in data evaluation.

The biological filters in each tank were preinoculated with a commercial preparation of nitrifying bacteria (Aqua-Gold, LaMonte Environmental Technology, Saticoy, CA), or by addition of filter matrix material from "mature" functioning filters. This insured that the biological filters were functional when the test organisms (the NTOs and the MPCA) were introduced. Artificial seawater (Forty Fathoms, Marine Enterprises, Towson, MD) was used in Trials 1 through 5; city tap water was used to make up the artificial seawater. City tap water was used directly in the freshwater Trials 6 and 7. Salinity, pH, ammonia (Kordon Aquatru Water Test Kit, Hayward, CA), nitrite (TetraTest Nitrite, TetraWerke, Ulrich Baensch, West Germany), and alkalinity (Model AL-AP Test Kit, Hatch Co., Loveland, CO) of the tank water were monitored and maintained (by partial water exchanges, manipulation of feeding rate, use of room space heaters/coolers, etc.) within the limits listed (Tables 7 and 8).

Table 7. Ranges of environmental parameters in test and control tanks during each MPCA trial run in seawater.

Trial	Water Temp. (°C)	pH	Salinity (ppt)	Ammonia (mg/L)	Nitrite (mg/L)	Total Alkalinity (mg/L $CaCO_3$)
1	19.5-22.0	7.9-8.0	25-27	0	0.15-0.6	-
2	23.5-26.0	8.2-8.4	24	0-0.2	0-0.1	205-221
3	21.0-24.0	7.9-8.2	25-28	0.1-0.2	0.1-0.4	187-255
4	22.5-25.0	8.2-8.5	24-27	0-0.8	0.1-2.5	157-255
5	21.0-23.0	7.8-8.7	25-32	0-0.2	0.1-0.25	119-306

Table 8. Ranges of environmental parameters in test and control tanks during each MPCA trial run in freshwater.

Trial	Water Temp. (°C)	pH	Disolved Oxygen (mg/L)	Ammonia (mg/L)	Nitrite (mg/L)	Total Alkalinity (mg/L $CaCO_3$)	Lighting (micro E)
6	18.5-23.0	7.2-8.2	5.5-11.0	0	0.1-0.5	136-170	0.45-2.4
7	18.0-24.5	7.6-8.9	5.2-6.8	0-2.4	0-0.5	136-187	1.2-3.0

Sampling Methods

Water--

Sterile pipets were used to collect approximately 5 ml of water from each control and test tank. The water was placed into sterile plastic tubes and kept on ice until it was brought to the lab. For microbiology assays, 0.1 ml was dropped into the middle of the appropriate media plate, spread with a sterile "hockey stick" (a bent glass rod) and placed in an incubator (30°C for P. putida and 37°C for B. sphaericus). For the B. sphaericus vegetative cell study, the plates were sprayed with catechol following overnight incubation. For gene probe assays, the water was stored at -20°C until the gene probe assay was performed.

Tissues--

NTOs were placed in plastic bags on ice once removed from the experimental tanks. At the lab, the organisms were surface sterilized by soaking them in Fritz's egg disinfectant (a 10% iodine solution; Fritz Chemical Co., Dallas, TX) for approximately 5-10 min. Oysters, mussels, snails and fish were scrubbed with a brush before being washed twice in sterile distilled water. The remaining organisms were also washed two times with sterile distilled water.

For the shrimp, fish, oysters, and mussels, the gills, intestines, and pieces of muscle were aseptically removed and placed in a sterile blender containing 20 ml of 0.01 M Tris buffer, pH=7.0. The tissues were then homogenized for 30 seconds. Plants, worms, shorefly larvae, and anemones were homogenized by hand using tissue homogenizers. One hundred microliters of each resulting homogenate were plated on duplicate plates of the appropriate media, and spread with a sterile hockey stick. The plates were then incubated. The remainder of the sample was stored at -20°C for subsequent gene probe assays or for storage. For the gene probe assay, 0.5 ml of each sample was added to 0.5 ml deionized formamide and incubated for 30 min at 80°C to liberate nucleic acid. The samples were then applied to a Gene Screen plus hybridization membrane, baked at 80°C for 2 hr, prehybridized, hybridized, washed and placed on X-ray film to produce an autoradiogram ("blot").

Histological Samples--

Samples for histological examination were preserved in Davidson's acetic acid formalin alcohol fixative (Humason, 1972) for 24 to 76 hr, transferred to 50% ethanol for storage, and later processed and examined using routine histological methods (Luna, 1968). Mayer's hematoxylin and phloxine/eosin stain (Sheehan and Hrapchak, 1980) was used for all NTO specimens. In addition, Brown and Brenn tissue Gram stain (Luna, 1968) was used in those trials in which bacterial model MPCAs were used.

Method for Obtaining Plasmid DNA for Gene Probes

For the studies using the MPCAs AcMNPV and P. putida (Tables 5 and 6), it was necessary to harvest plasmid DNA for labeling as gene probes. Both plasmids originated from the pUC 18 family of plasmids and were placed in E. coli strains, and both plasmids contained an Ampicillin resistance gene. To harvest large amounts of the DNA, the appropriate E. coli was grown in LB media supplemented with 30-50 ug/ml Ampicillin. Overnight cultures were pelleted, the bacteria washed and the plasmid was isolated using the alkaline lysis procedure (Maniatis et al., 1982).

TRIALS WITH MODEL MPCAs

Background Studies

Background studies were conducted before beginning trials with model MPCAs in the marine multispecies test tanks. The NTOs were examined histologically to determine what major parasites and/or obvious infectious pathogens might be present (and later encountered in trials with model MPCAs). Tables 9 and 10 list those parasites, pathogens, symbionts and lesions found to occur naturally in the marine and freshwater NTOs. In addition to the histological studies, background microbiological and gene probe studies were run with NTO tissue homogenates prepared in phosphate buffer (0.17 M KH_2PO_4, 0.7 M K_2HPO_4, Mallinckrodt, Inc., Paris, KY); test tank seawater was split: half was seeded with the model MPCA, while the other half (controls) were not seeded. Recovery of the model MPCAs from the tissue homogenates and tank water was attempted to insure that no interfering substance would cause false positives or negatives.

General Sampling Scheme

Three test tanks were inoculated on day 0 of a planned 28 to 30 day study with the model MPCAs to dose levels listed in Table 11. The water was then mixed for 5 min, and water samples were taken from each tank to determine initial concentration of recoverable model MPCA. At predetermined time intervals throughout the study, water and NTO samples were collected for microbiological and histological analyses (Table 12).

For the trials using B. sphaericus spores, a large quantity of spores was received from Dr. A. Youston and stored at 4°C. The spore content of this stock was titered by plate count on TBAB plates containing 100 ug/ml streptomycin and 5 ug/ml Neomycin. An appropriate volume of spore suspension to achieve the dose rate representative of that used in field applications (~10^{5-6} CFU/ml; Davidson et al., 1984) was then added to the test tanks (Table 11).

Table 9. Summary of histological observations of lesions, parasites, and other anomalies present in the marine non-target organisms (NTOs) used in MPCA Trials 1 through 5, that were unrelated to treatment effects of the MPCAs tested.

Marine NTO Species	Parasites or Lesions Observed
Sea anemone	None observed.
Turbin snail	Rickettsia in gill epithelial cells; Probable haplosporidan in gills; Probable coccidian in gut mucosa; Ciliate protozoan on gills.
Japanese oyster	Generalized atrophy of tissues in some batches used.
Estuarine grass shrimp	Gregarines in midgut and hepatopancreas; Encysted larval cestodes in various tissues resulting in large granulomas.
Shore fly larvae	None observed.
Sheepshead minnow	Microsporidan cysts in brain; Thyroid goiters; Gill hyperplasia, clubbing, capillary aneurysms and lamellear fusion; Excessive hemosiderin, lipid and/or glycogen accumulation in liver.
Salicornia	Yellowing, browning and wilting of hydroponically grown (estuarine plant) plants.

Table 10. Summary of histological observations of lesions, parasites, and other anomalies present in the freshwater non-target organisms (NTOs) used in MPCA Trials 6 and 7, that were unrelated to treatment effects of the MPCAs tested.

Freshwater NTO Species	Parasites or Lesions Observed
Tubifex worm	Multinucleate protozoan in hemocoel; Surface fouling by blue green algae; Large basophilic cytoplasmic inclusion in some individuals (viral, chlamydial ?).
Snail	None observed.
Freshwater mussel	Massive storage of grey-blue mineral granules in loose connective tissues.
Grass shrimp	Parasitic nematode in gut; Massive systemic infection by a rickettsia or chlamydia.
Sailfin molly	Gill epithelium hyperplasia and lamellar fusion.
Anacharis plant	Surface fouling of thallus by blue green algae, rotifers, diatoms, bacteria, and ciliated protozoans.

Table 11. Dose levels of model MPCAs used in Trials 1 through 7*.

Trial Number	Model MPCA	Initial Dose Level
1 (M)	Bacillus sphaericus spores	10^6 CFU/ml
2 (M)	B. sphaericus vegetative cells	2×10^6 CFU/ml
3 (M)	AcMNPV baculovirus occlusions	10^6 occlusion bodies/ml
4 (M)	Pseudomonas putida	10^6 CFU/ml
5 (M)	B. sphaericus spores	1.6×10^7 CFU/ml
6 (FW)	P. putida	7.5×10^4 CFU/ml
7 (FW)	AcMNPV baculovirus occlusions	1.8×10^5 occlusion bodies/ml

* M = test run in saltwater with marine NTO species.
FW = test run in freshwater with FW NTOs.
CFU = colony forming units.

Table 12. General sampling scheme used in Trails 1 through 7 with model MPCAs.*

Day of Trial	Non-target Test Organism						Tank Water
	Snail	Fish	Worm	Bivalve	Shrimp	Plant	
0	M,H	M,H	M,H	M,H	M,H	M,H	M,C
i	---------- not done ----------						M
1	M	M	M	M	M	M	M,C
7	M	M	M	M	M	M	M,C
14	M,H	M,H	M,H	M,H	M,H	M,H	M,C
21	M	M	M	M	M	M	M
30	M,H	M,H	M,H	M,H	M,H	M,H	M,C

* M = samples for microbiological and/or gene probe tests.
H = samples for histological analysis.
C = samples for "chemical" water quality analyses.
i = "initial;" sample taken immediately after MPCA added.

B. sphaericus vegetative cells were inoculated into 1 L of brain heart infusion broth supplemented with 5 ug/ml Neomycin and 50 ug/ml thymine for Trial 2. The cells were pelleted, resuspended in Tris buffer, and plated on TBAB agar with 5 ug/ml Neomycin and 50 ug/ml thymine. The following day, plates were sprayed with catechol (0.55 g/ml) and colonies counted to determine the volume needed for addition to the test tanks.

For Trials 3 and 7, the concentration of the stock of AcMNPV occlusion bodies was estimated using a hemocytometer. The the appropriate amount to be added to the test tanks was determined.

In Trials 4 and 6, Pseudomonas putida was grown in 1 liter of LB media supplemented with 500ug/ml nalidixic acid and 150 ug/ml Kanamycin. Cells were then pelleted, resuspended in Tris buffer and plated on PsF Agar supplemented with 500 ug/ml nalidixic acid and 150 ug/ml Kanamycin. The titer was determined by total plate count, and the appropriate amount added to the test tanks.

Trial 1: Bacillus sphaericus 2362 with pLT103; Spores

The model MPCA, B. sphaericus spores, for Trial 1 was recoverable from the test tank water throughout the 28 day study (Table 13). Water and NTOs from the control tanks were positive

on days 7 and 14. for this MPCA as well. The contamination was at a low level and was not detected after day 14. For subsequent studies test tanks were then covered, moved, and physically separated from control tanks to prevent aerosol contamination of the model MPCA.

B. sphaericus-like spores and vegetative cells were noted in the gut contents of some of the test animals examined histologically (Figures 9-11). Presumably, these Gram positive large rods and spores were consumed by the NTO. However, despite their presence in the gut contents of these animals, no lesions, inflammation, reduced survival, or other signs of infection or toxicity accompanied their presence (Tables 14 and 15).

Trial 2: Bacillus sphaericus 1593 with pLT117; Vegetative Cells

B. sphaericus vegetative cells, which expressed the xylE gene for catechol 2,3-dioxygenase, were used as the model MPCA in Trial 2. The vegetative cells did not persist in the test system and were recoverable by microbiological assay only immediately after the test tanks were seeded (Table 16). Similarly, gross signs, survival, and histological study of control and MPCA exposed NTOs showed no differences, and no adverse effects attributable to the MPCA (Tables 14 and 15).

Trial 3: Baculovirus AcMNPV; Inclusion Bodies

A gene probe to the polyhedrin gene of baculovirus AcMNPV was used in Trial 3 in an attempt to track the fate and persistence of this viral MPCA. Preliminary tests and controls run with saltwater and NTO tissues "spiked" with AcMNPV inclusion bodies showed detection of the virus above background levels, but positive signals were quite weak. In addition, control tissues from the minnow showed some weak but non-specific binding of the probe (Figure 12). Despite the weakness of the probe and because of the absence of an alternative detection method, this trial was run with the gene probe as the detection method.

The results of this trial indicated that the virus could be detected in the test tank water using the gene probe on the initial day of the seeding of the water (Figure 13). On subsequent days of the study, however, the baculovirus could not be detected. The control tissues of the NTO's produced negative results also. For the test tissues, the larval shore fly and plant tissues produced very faint signals that were only slightly more intense than the background blot (Figure 12). Therefore, either the baculovirus did not persist in the test tanks' seawater or in the tissues of the NTOs beyond day 1 of the trial, or the probe lacked adequate affinity to the viral DNA to demonstrate its presence. In either case, gross signs, survival, and histological study of control and MPCA exposed NTOs showed no differences and no adverse effects attributable to the MPCA (Tables 14 and 15).

Table 13. Summary of microbiological assays for Bacillus sphaericus (spores) in Trial 1.

Day of Trial	CONTROLS NTOs F	GS	O	S	Tank Water 8	9	10	EXPOSED NTOs F	GS	O	S	Tank Water 1	2	3
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
i	--------	nd			----------			----	nd		----	+	+	+
1	--------	nd			----------			----	nd		----	+	+	+
7	+	+	+	+	+	+	+	+	0	+	+	+	+	+
14/15	0	0	+	+	0	0	0	+	+	+	+	+	+	+
21/22	0	0	0	0	0	0	0	+	+	+	+	+	+	+
28/29	0	0	0	0	0	0	0	+	+	+	+	+	+	+

Abbreviations used:
0 = MPCA not recovered from the test system water or NTOs.
+ = MPCA recovered from test system water or NTOs.
i = sample taken immediately after MPCA introduced.
F = sheepshead minnow (fish)
GS = grass shrimp
S = turbin snail
O = oyster
nd = not done

Table 14. Initial number, observed mortalities, and adjusted percent survival* of selected NTOs following exposure to model MPCAs in seawater Trials 1-5 and freshwater Trials 6-7.

	Shrimp		Minnow		Snail		Oyster	
Trial	T	C	T	C	T	C	T	C
Trial 1:								
Initial No.	45	45	45	45	45	45	45	45
No. sampled	26	27	26	27	28	26	28	26
Mortalities	1	1	4	3	0	1	0	0
Survivors	12	9	10	12	16	17	15	20
% survival	84	80	80	87	98	96	96	102
Trial 2:								
Initial No.	45	45	45	45	45	45	45	45
No. sampled	18	18	18	18	18	18	18	18
Mortalities	3	1	2	1	1	4	6	2
Survivors	25	20	32	31	28	29	27	27
% survival	96	84	111	108	102	104	100	100
Trial 3:								
Initial No.	45	45	45	45	45	45	45	45
No. sampled	22	21	22	21	22	21	24	20
Mortalities	1	0	2	2	0	0	1	5
Survivors	9	13	16	14	16	20	16	19
% survival	69	76	84	78	84	91	89	87
Trial 4:								
Initial No.	45	45	45	45	45	45	45	45
No. sampled	18	10	18	11	18	10	19	10
Mortalities	0	0	6	2	2	2	2	3
Survivors	20	31	21	26	27	33	21	32
% survival	84	91	86	82	100	96	89	93
Trial 5:								
Initial No.	45	45	45	45	45	45	45	45
No. sampled	22	19	21	19	21	19	21	19
Mortalities	2	1	3	10	3	4	1	0
Survivors	16	14	17	8	13	13	24	28
% survival	84	73	84	60	76	71	100	104
Trial 6:								
Initial No.	45	45	45	45	45	45	45	45
No. sampled	28	12	28	12	28	12	28	12
Mortalities	1	0	0	0	0	0	0	2
Survivors	2	16	16	28	13	15	16	30
% survival	66	62	98	89	91	60	98	93
Trial 7:								
Initial No.	75	75	45	45	45	45	45	45
No. sampled	18	14	18	14	18	14	18	14
Mortalities	19	3	0	4	0	0	0	0
Survivors	12	26	29	24	30	29	31	25
% survival	40	53	104	84	107	96	109	87

* Adjusted % survival = 100 x [(number survivors + number sampled) / (initial number)].

Table 15. Summary of observations in which organisms, presumed to be the MPCA being tested, were observed in histological sections of marine non-target test organisms.

	MPCA TESTED (in Trials 1-5)*			
Marine NTO Species	Bacilius Spores(1)	Rods(2)	Pseudomonas(4)	AcMNPV(3)
Sea anemone	- *	-	-	-
Turbin snail	GC**	GC	-	-
Japanese oyster	GC	GC	-	-
Estuarine grass shrimp	GC	GC	-	-
Shore fly larvae	GC	GC	-	-
Sheepshead minnow	GC	-	-	-
Salicornia (estuarine plant)	-	-	-	-

* - = not detected in or on any tissue examined.

**GC = "gut contents": signifying that organisms were present in the gut contents that were identical morphologically to the MPCA tested. Other than in the gut contents, no organisms were observed in the tissues of NTOs with morphologies similar to the MPCAs tested.

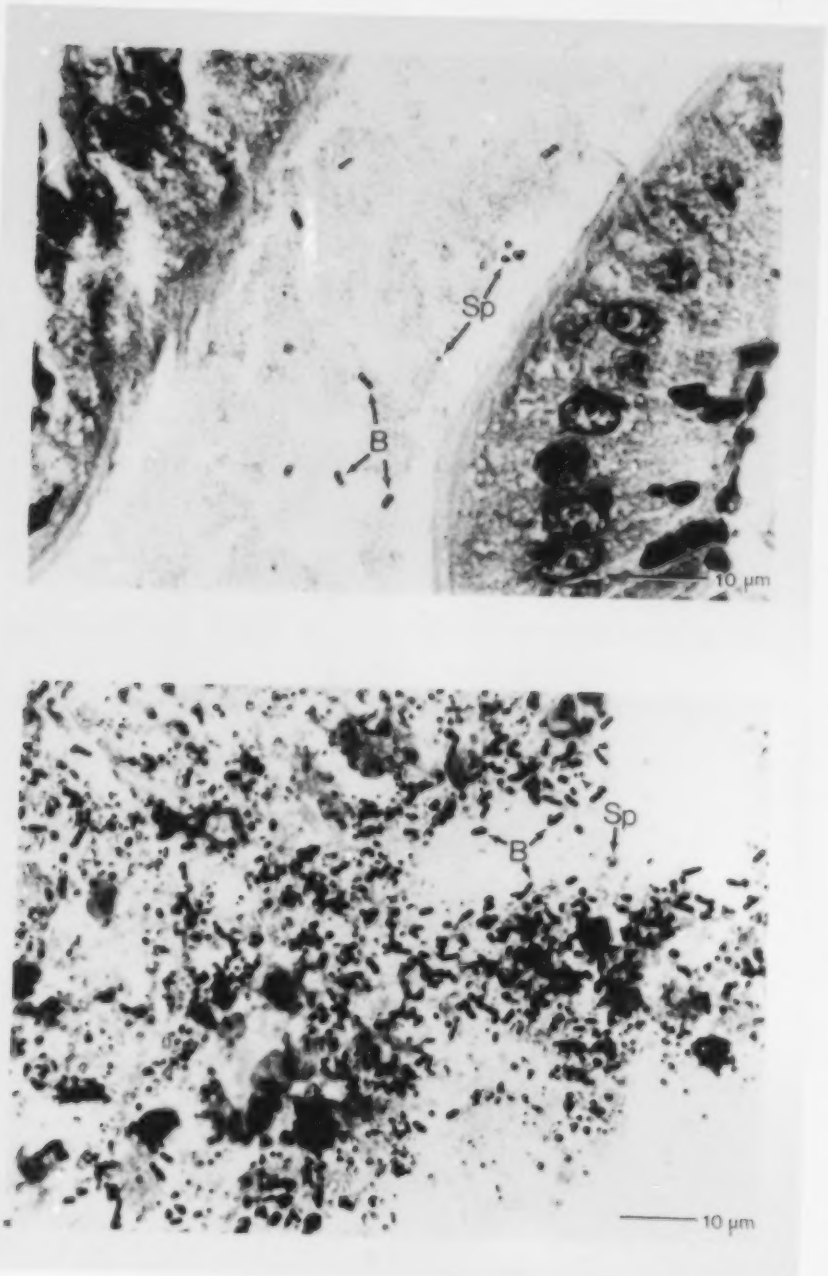


Figure 9. Light photomicrograph of normal sheepshead minnow gut showing presumed Bacillus sphaericus spores (SP) and bacilli (B). Brown-Brenn Gram stain. Bar = 10u.

Figure 10. Light photomicrograph of normal snail gut showing presumed Bacillus sphaericus spores (SP) and bacilli (B). Brown-Brenn Gram stain. Bar = 10u.

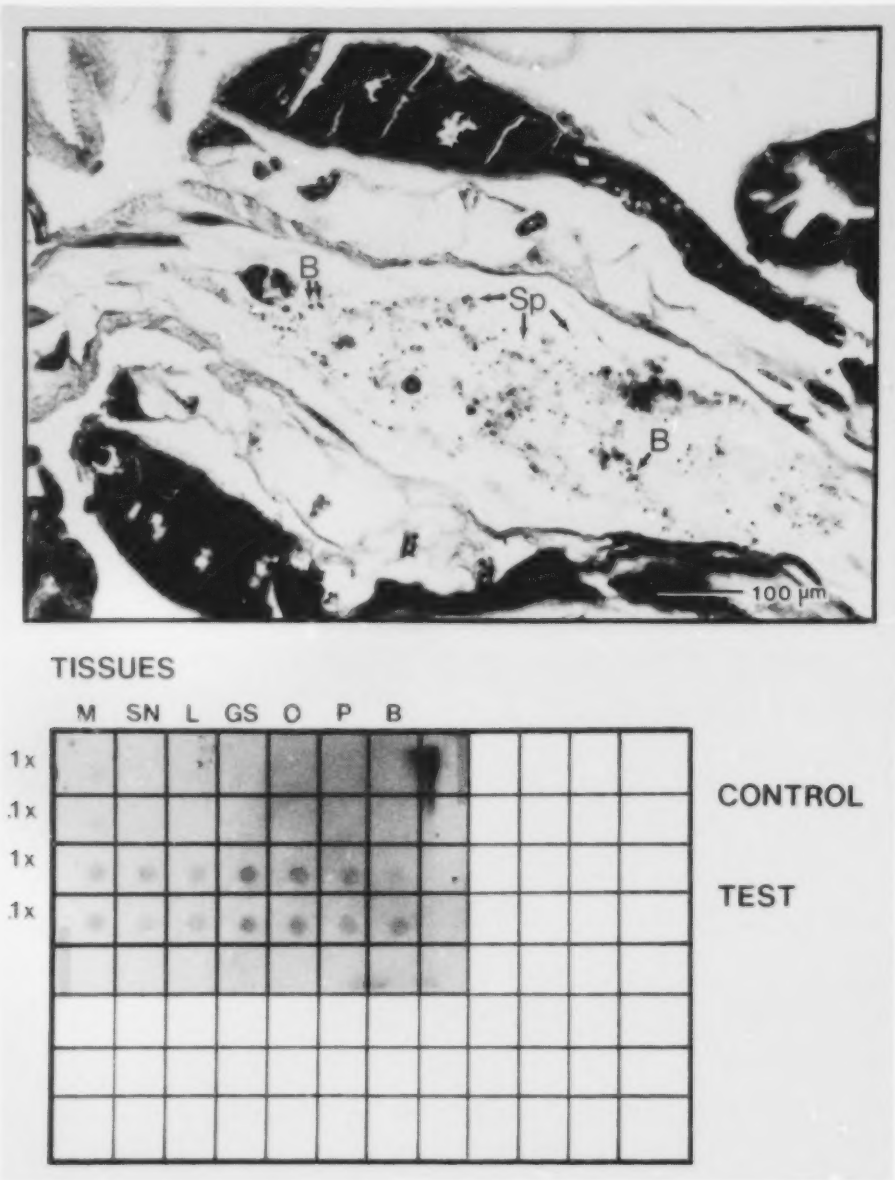


Figure 11. Light photomicrograph of normal shore fly gut showing presumed Bacillus sphaericus spores (SP) and bacilli (B). Brown-Brenn Gram stain. Bar = 100u.

Figure 12. Autoradiograph showing the results of the gene probe assay for the detection of AcMNPV in seeded (S) and unseeded (US) non-target organism tissues. Samples were applied to the membrane undiluted (1X) and diluted 1:10 (.1X). M=minnow, SN=snail, L=shore fly larvae, GS=grass shrimp, O=oyster, P=plant, and B=buffer (negative control).

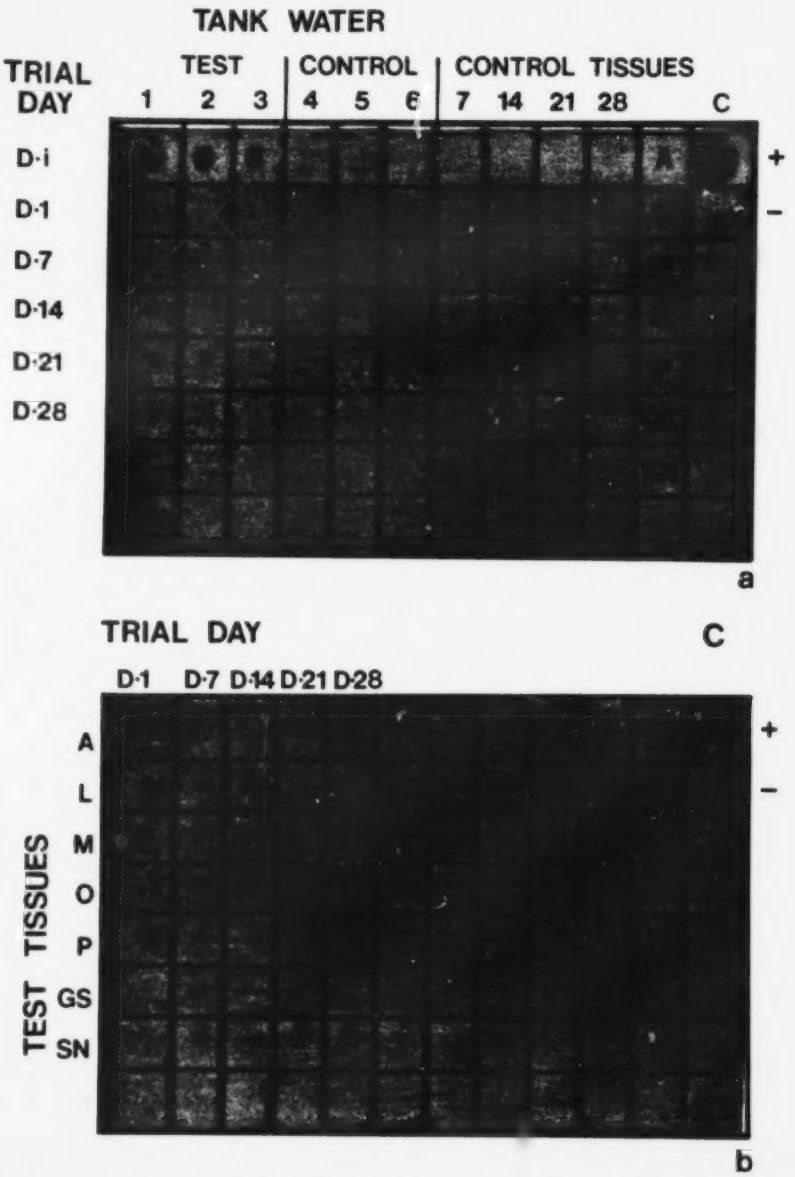


Figure 13. Autoradiograph showing the results of the gene probe assay for the detection of AcMNPV in non-target organism tissues and tank water for Trial number 3. a) Results of test and control tank water and control non-target organism tissues. b) Results of test non-target organism tissues. A=anenome, M=minnow, O=oyster, P=plant, GS=grass shrimp, L= shore fly larvae, SN=snail, C=control, + = positive control (unlabeled plasmid DNA), and - = negative control (buffer).

Table 16. Summary of microbiological assays for Bacillus sphaericus (vegetative cells) in Trial 2.

Day of Trial	CONTROLS NTOs F	GS	O	S	P	D	Tank Water 9	10	11	EXPOSED NTOs F	GS	O	S	P	D	Tank Water 1	2	3
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
i	-------- nd ----------									---- nd ----						+	+	+
1	-------- nd ----------									---- nd ----						0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Abbreviations used:
0 = MPCA not recovered from the test system water or NTOs.
+ = MPCA recovered from test system water or NTOs.
i = sample taken immediately after MPCA introduced.
nd = not done
F = sheepshead minnow (fish)
GS = grass shrimp
O = oyster
S = snail
P = Salicornia root (plant)
D = shore fly larvae (dipteran)

Trial 4: Pseudomonas putida PPO200 + pEPA74

The results from two detection methods used to determine the persistence of this model MPCA in Trial 4 correlated well (Table 17; Figure 14). Both the gene probe method and microbiological method showed that the MPCA could not be detected after day 2 of the study in either the tank water or the NTO tissues. The pseudomonad was detected in the oyster tissues on day 1 and day 2 and in the anemone tissues on day 2. Control tissues and water samples were clearly negative. Similarly, gross signs, survival, and histological study of control and MPCA exposed NTOs showed no differences and no adverse effects attributable to the MPCA (Tables 14 and 15).

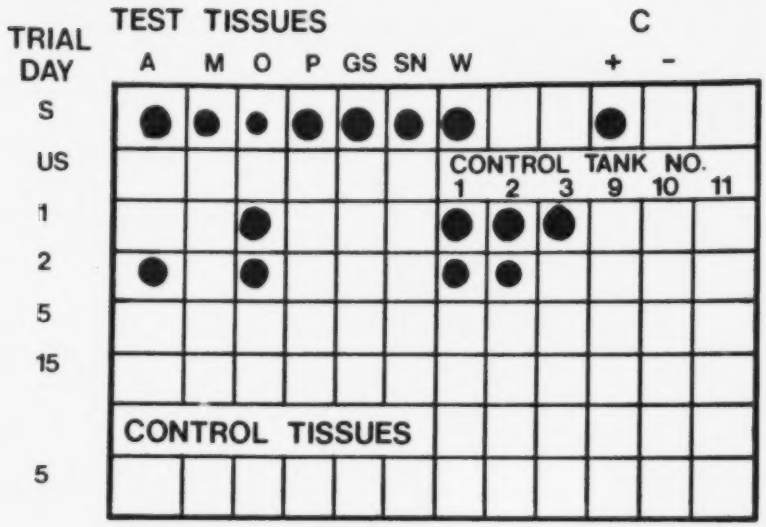


Figure 14. Autoradiograph showing the results of the gene probe assay for the detection of Pseudomonas putida in seeded (S) and unseeded (US) non-target organism tissues and non-target organism tissues from Trial 4. Control tissues were only tested on day 5 of the study. 1,2,3=test tank numbers, 9,10,11= control tank numbers, A=anenome, M=minnow, O=oyster, P=plant, GS=grass shrimp, SN=snail, W=tank water, C=control, + = positive control (unlabeled plasmid DNA), and - = negative control (buffer).

Table 17. Summary of microbiological assays for Pseudomonas putida in Trial 4.

Day of Trial	CONTROLS NTOs F	GS	O	S	A	P	Tank Water 9	10	11	EXPOSED NTOs F	GS	O	S	A	P	Tank Water 1	2	3
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
i	-------- nd ----------									---- nd ----						+	+	+
1	-------- nd ----------									0	0	+	0	0	0	+	+	+
2	---- nd ----						0	0	0	0	0	+	0	+	0	+	+	0
5	--------- nd ---------									0	0	0	0	0	0	0	0	0
15	--------- nd ---------									0	0	0	0	0	0	0	0	0

Abbreviations used:
0 = MPCA not recovered from the test system water or NTOs.
+ = MPCA recovered from test system water or NTOs.
i = sample taken immediately after MPCA introduced.
F = sheepshead minnow (fish)
GS = grass shrimp
S = turbin snail
O = oyster
A = sea anemone
P = Salicornia sp. (plant)
nd = not done

Trial 5: Bacillus sphaericus 2362; Spores

This model MPCA was shown to persist throughout Trial 5 in all water and NTO test tissue samples (Table 18). In the test tank, concentration of viable spores in the seawater at the end of the study remained relatively high, at approximately 10^4 CFU/ml. Unlike the disconcerting findings of Trial 1, in which this model MPCA was detected in the control tanks, it was not detected in the control samples assayed.

As was the case in Trial 1, B. sphaericus-like spores and vegetative cells were noted in the gut contents of some test animals examined histologically. Presumably, these Gram positive large rods and spores were consumed by some of the NTOs. However, despite their presence in the gut contents of these animals, no lesions, inflammation, reduced survival, or other signs of infection or toxicity accompanied their presence (Tables 14 and 15).

Table 18. Summary of microbiological assays for Bacillus sphaericus spores in Trial 5.

Day of Trial	CONTROLS NTOs F	GS	O	S	P	A	Tank Water 9	10	11	EXPOSED NTOs F	GS	O	S	P	A	Tank Water 1	2	3
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
i	-------- nd ----------									---------- nd ----------								
1	0	0	0	0	0	0	0	0	0	+	+	+	+	+	+	+	+	+
5	---- nd ----						0	0	0	+	+	+	+	+	+	+	+	+
12	0	0	0	0	0	nd	0	0	0	+	+	+	+	+	+	+	+	+
15	0	0	0	0	0	0	0	0	0	+	+	+	+	+	nd	+	+	+
30	0	0	0	0	0	nd	0	0	0	+	+	+	+	+	+	+	+	+

Abbreviations used:
0 = MPCA not recovered from the test system water or NTOs.
+ = MPCA recovered from test system water or NTOs.
i = sample taken immediately after MPCA introduced.
F = sheepshead minnow (fish)
GS = grass shrimp
S = turbin snail
O = oyster
P = Salicornia sp. (plant)
A = sea anemone
nd = not done

Note: Concentration of MPCA spores at day 30 was 10^4 CFU/ml, down from the initial dose of 1.6×10^7 CFU/ml.

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Trial 6: Pseudomonas putida PPO200 + pEPA74

Microbiological results from Trial 6 showed that the MPCA could be isolated from test system water and from the tissues of certain NTOs for the first several days of the trial. For the remainder of the 29-day trial, detection of the MPCA was sporadic and in low numbers in exposed NTOs' tissues (Table 19). The data are also represented graphically in Figures 15 and 16. Interestingly, the results show that the tubifex worms, freshwater snails, and the mollies harbored the MPCA, in just detectable amounts, probably for the duration of the trial. In contrast, the pseudomonad appeared to be cleared from the test tanks' water by day 7. In view of these findings, one might

speculate that this pseudomand had colonized certain of the NTOs, becoming part of their microflora. All control organisms and water samples were negative for the presence of P. putida throughout the trial (Figure 16).

The results of the gene probe study did not correlate well with the microbiological assay results (Figure 17). Gene probe results showed cross reactivity with something found in the tissues and water of the test system. Tissue and water samples from control and test tanks demonstrated positive results at day 0, even prior to the addition of the MPCA to the test tanks. In Figure 17 the "+" sign on the blots indicates a positive control consisting of unlabeled plasmid DNA. This shows that the DNA for the gene probe had been labeled properly and hybridization did occur. This means that the other positive signals shown in Figure 17 may represent the presence of other microorganisms in the test system and NTOs with homologous nucleic acid sequences.

As in Trial 4, in which this MPCA was tested with marine NTOs, gross signs, survival, and histological study of control and MPCA exposed NTOs showed no differences and no adverse effects attributable to the MPCA (Tables 14 and 20).

Trial 7: Baculovirus AcMNPV; Occlusion Bodies

The baculovirus model MPCA in Trial 7 may have persisted in some of the test samples. The most intense gene probe signal was seen at day 30 in the test tank water (Figure 18). However, non-specific binding of the probe was clearly indicated in this study. This was evident especially at the cut borders of the blot, and in day 0 signals that provided false positives when they should have been negative (Figure 18). Therefore, as was concluded in Trial 3 run with this MPCA in seawater, the results of Trial 7 indicated that either the baculovirus did not persist in the test tanks' water or in the tissues of the NTOs beyond day 1 of the trial, or that the probe lacked adequate affinity to the viral DNA to demonstrate its presence. While AcMNPV occlusion bodies were observed in the ingested gut contents of snails in Trial 7, gross signs, survival, and histological study of control and MPCA exposed NTOs showed no differences and no adverse effects attributable to the MPCA (Tables 14 and 20).

Table 19. Summary of microbiological assays for Pseuodmonas putida in Trial 6.

Day of Trial	MPCA EXPOSED* NTOs W	S	F	M	GS	P	Tank Water 4	5	6
0	0	0	0	0	0	0	0	0	0
i	---------- nd ----------						$1x10^4$	$9x10^3$	$1x10^4$
1	200	10^3	10^4	80	10^3	10	$1x10^3$	$4x10^3$	$6x10^3$
2	---------- nd ----------						0	700	0
4	240	10^3	290	50	0	0	0	100	0
7	0	60	70	10	0	0	0	0	0
12	0	140	60	0	0	0	0	0	0
15	nd	0	0	0	0	0	nd	nd	nd
20	20	20	0	0	0	0	0	0	0
29	nd	0	60	0	0	0	0	0	0

* Units shown are MPCA CFU/ml.
Control samples assayed from days 0, 11, and 29 of the study were uniformly negative for the MPCA.

Abbreviations used:
0 = MPCA not recovered from the test system water or NTOs.
i = sample taken immediately after MPCA introduced.
W = tubifex worm
S = freshwater snail
F = sailfin molly (fish)
M = freshwater mussel
GS = freshwater grass shrimp
P = anacharis plant
nd = not done

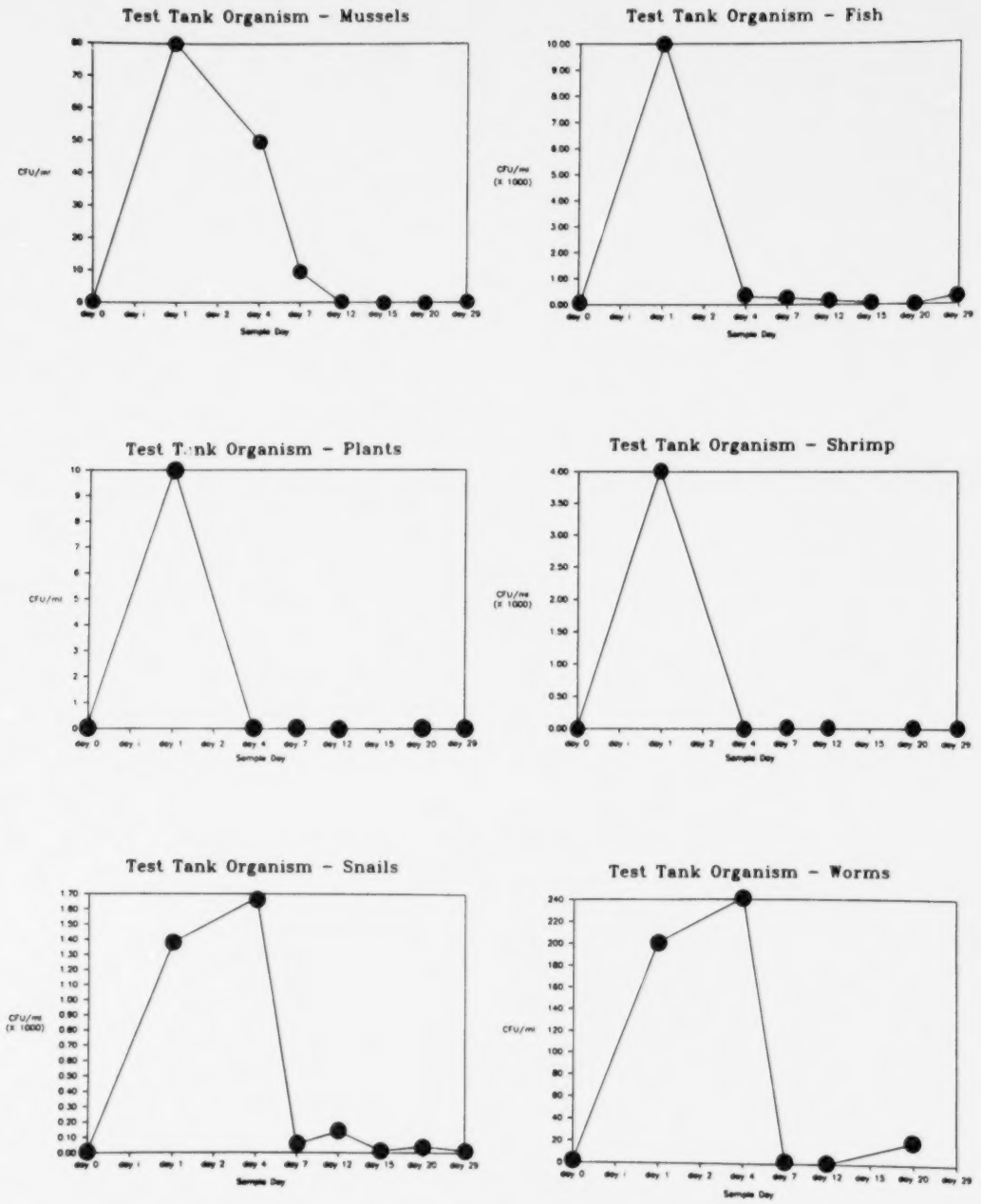
Test Tank Organism - Mussels
Test Tank Organism - Fish
Test Tank Organism - Plants
Test Tank Organism - Shrimp
Test Tank Organism - Snails
Test Tank Organism - Worms
Sample Day

Figure 15. Graphs representing the microbiological results for the detection of Pseudomonas putida in non-target organism tissues from test tanks from Trial 6. Results from non-target control organisms are not represented since the results showed no P. putida present in control samples.

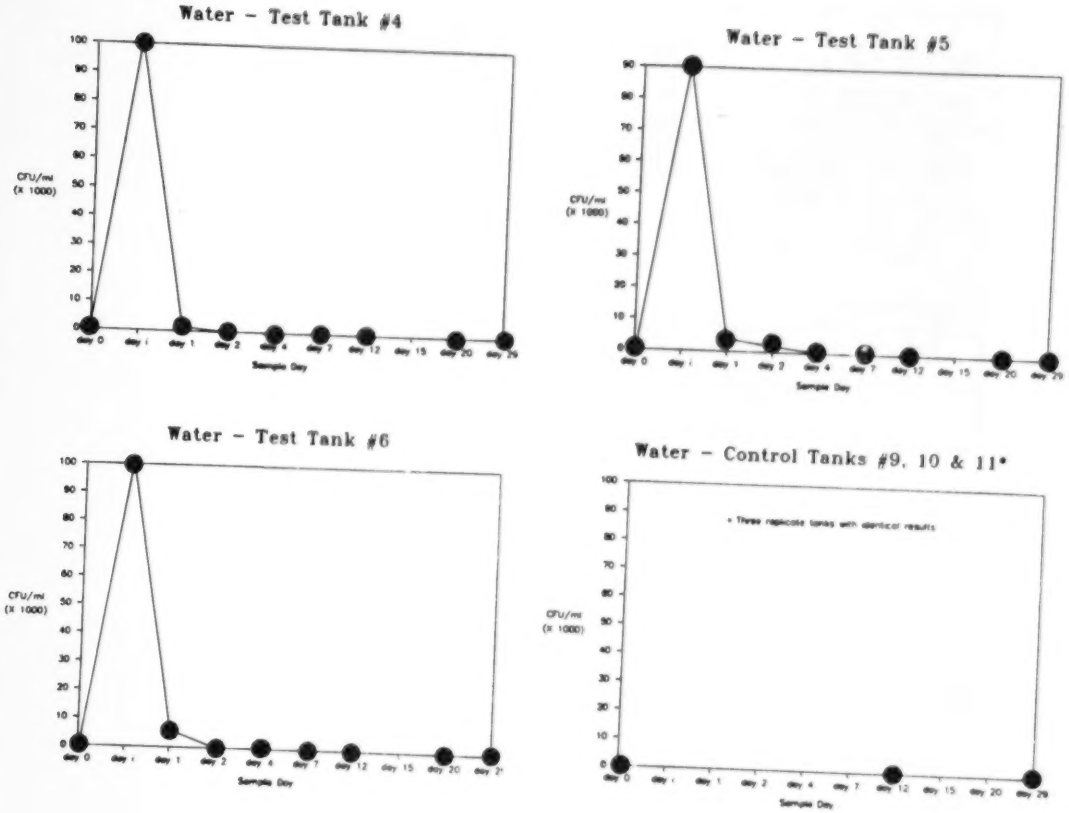
Water - Test Tank #4
Water - Test Tank #5
Water - Test Tank #6
Water - Control Tanks #9, 10 & 11*
CFU/ml
(X 1000)
Sample Day
* Three replicate tanks with identical results

Figure 16. Graphs representing the microbiological results for the detection of Pseudomonas putida in water samples from the test and control tanks used in Trial 6.

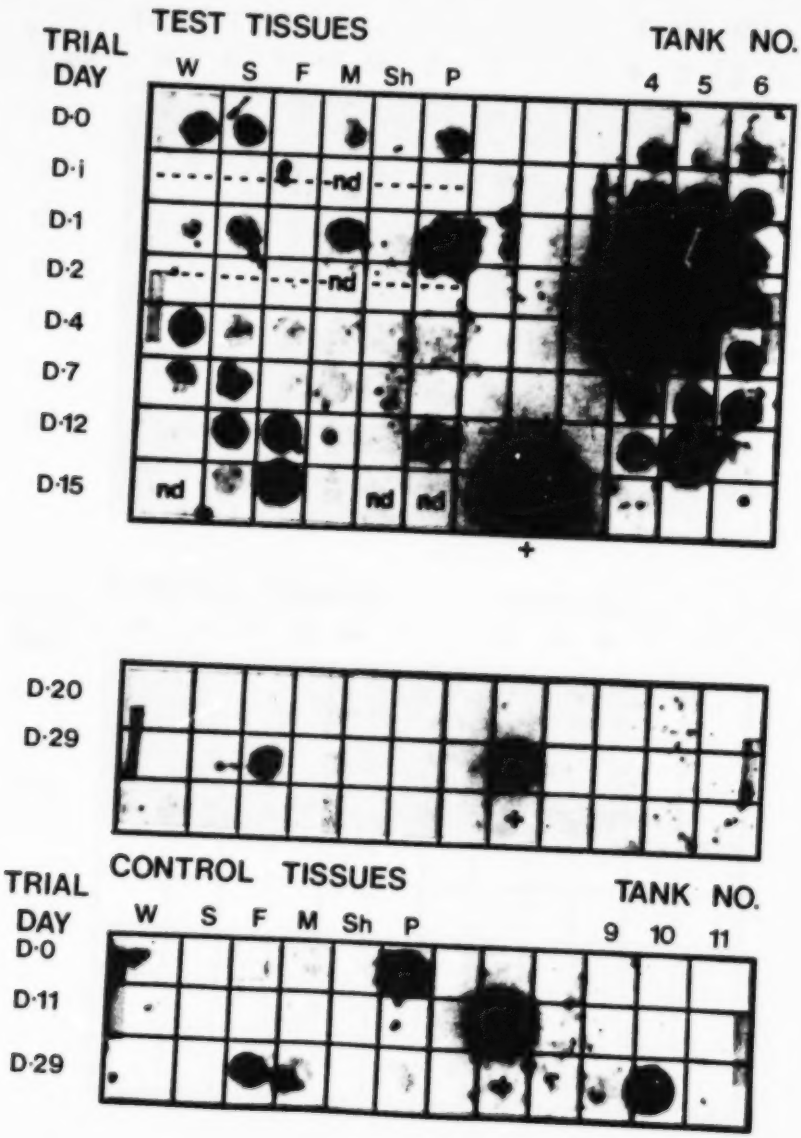

Figure 17. Autoradiographs showing the results of the gene probe assay for the detection of Pseudomonas putida in non-target tissues and tank water for Trial 6. W=tubifex worm, S=snail, F=fish, M=mussel, Sh=shrimp, P=plant, nd=not done, + = positive control (unlabeled plasmid DNA. Control samples were taken only on day 0, 11, and 29 of the study.

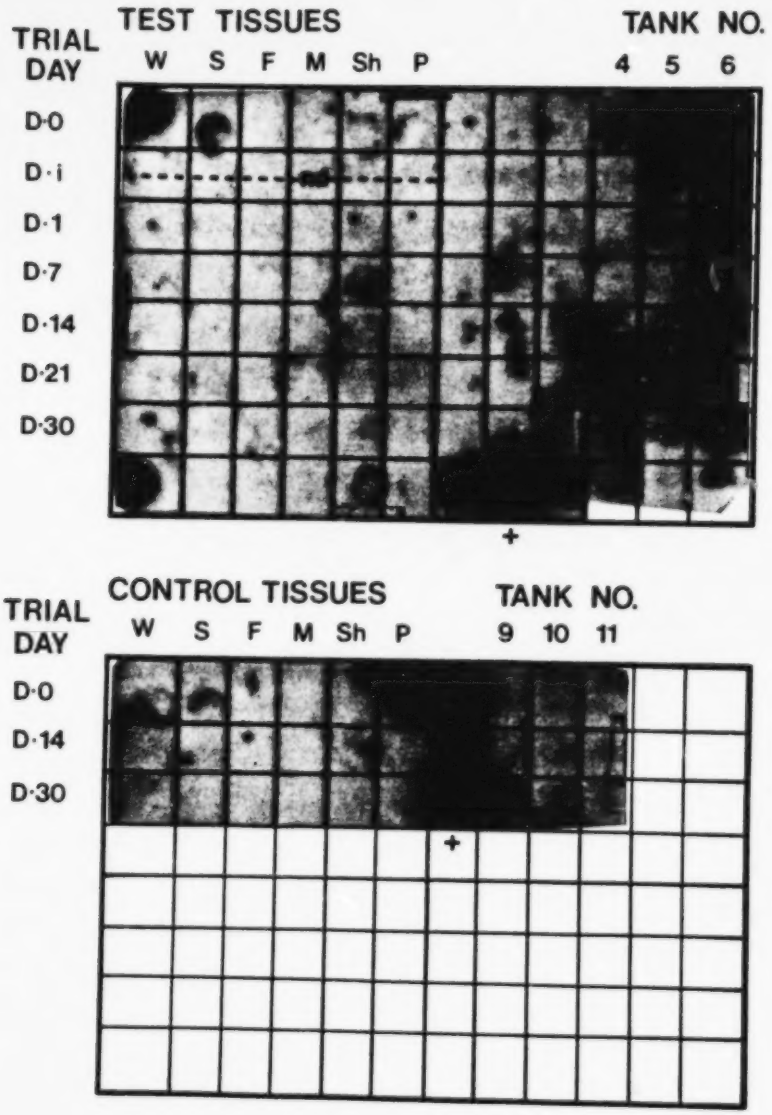

Figure 18. Autoradiographs showing the results of the gene probe assay for the detection of AcMNPV in non-target organism tissues and tank water for Trial number 7. W=tubifex worm, S=snail, F=fish, M=mussel, Sh=shrimp, P=plant, + = positive control (unlabeled plasmid DNA). Control samples were taken only on day 0, 14, and 30 of the study.

Table 20. Summary of observations in which organisms, presumed to be the MPCA being tested, were observed in histological sections of freshwater non-target test organisms.

Freshwater NTO Species	MPCA TESTED (in Trials 6 and 7)*	
	Pseudomonas (6)	Baculovirus (7)
Tubifex worm	GC**	-
Snail	GC	GC
Freshwater Mussel	GC	-
Grass Shrimp	-	-
Sailfin Molly	-	-
Anacharis plant	-	-

* - = not detected in or on any tissue examined.

** GC = "gut contents": signifying that organisms were present in the gut contents that were identical morphologically to the MPCA tested. Other than in the gut contents, no organisms were observed in the tissues of NTOs with morphologies similar to the MPCAs tested.

DISCUSSION

TEST SYSTEMS

An enclosed test system was developed in which multiple species of aquatic animals and plants can be tested for adverse non-target effects following experimental exposure to wild-type and genetically altered microbial pest control agents. Beginning with a test tank configuration similar in size and design to that described by Fournie et al., 1987; 1988), a variety of other possible tank sizes and configurations were constructed and tested as test systems for evaluation of MPCAs on non-target aquatic species. In all we developed and tested four additional tank configurations, which differed in construction materials, size, water circulation methods, and in configuration and type of biological filters used.

Each test system that we constructed had its own advantages and disadvantages. Tank systems No. 2 and 3, for example, were large tanks (1,000 L and 400 L, respectively) in which a very large number of species and individuals of NTOs could be tested with a model MPCA, while unplanned interspecific predation was controlled. However, the high cost of obtaining the relatively large amount of model MPCA required to perform a test in three replicate tanks of 400 to 1,000 L each prohibited their use in our studies. Therefore, we combined what we saw as the best features of our Test Systems 2 and 3 with those of the EPA system (Fournie et al. 1988) in designing Test Systems 4 and 5. Because of its simplicity in design, construction, and use, we utilized Test System 5 as our model multispecies test system in Trials 1-7 performed with model MPCAs that are representative of those being developed for possible use in the United States.

NON-TARGET SPECIES

A number of marine, estuarine, and freshwater animal and plant species were collected and evaluated for possible use as non-target species in multispecies test systems with wild-type and genetically altered MPCAs. Some proved to be excellent experimental species in terms of their availability, ease of laboratory culture, and representation of important phylogenetic groups in aquatic ecosystems. Others, while important ecologically, were not used as test animals and plants in our test system (Tables 1-3).

While no adverse effects were noted in some NTOs as a result of exposure to model MPCAs (i.e. in terms of survival, gross appearance, and histology of control and exposed specimens), data from the sea anemone, saltwater plant, and shore fly larvae were difficult to interpret due to problems with their use in the enclosed aquaria. The sea anemones moved between sampling times, making them difficult to find, and, therefore, they were not sampled during each scheduled sampling period.

Nematocyst filaments of the sea anemone stained Gram positive and fragments of these in histological sections were so similar in size to Bacillus vegetative rods as to be difficult to distinguish from the Gram positive bacilli used as the model MPCA. The saltwater plant we used in Trials 1-5 lost vitality during the trials, browned, and wilted. Salicornia is an estuarine plant and may require better lighting conditions or higher nutrient levels than were possible in the aquaria water in which the plants were grown hydroponically. The shore fly larvae were difficult to study as they pupated and adults emerged usually well before the end of a 28 day trial.

Of the freshwater species listed in Table 2, only the tubifex worms presented problems in their use as NTOs. Tubifex worms did not survive until the end of either of the freshwater experiments. It appeared that algae (mostly a filamentous blue green, probably Schizothrix calcicola and certain diatom species) colonized the exposed surfaces of the worms and overwhelmed them. By the last day of Trial 6, the netted bag containing the worms was coated with algae. In addition, the worms lived as a matted ball in their net container, making it difficult to pull apart a sample without damaging many of the worms. They were also more difficult to surface sterilize with iodine and rinse prior to microbiological assays. Because they were so fragile, they had to be processed separately from the other organisms. For Trial 7 we started with a larger mass of worms, but again, by the end of the study the worms were overwhelmed with algae. In nature tubifex worms dwell embedded in bottom sediments, which protect them from light and surface fouling organisms. If tubifex worms could be protected from predation in a multispecies test system, while being provided with a substrate in which to burrow, they may make an excellent NTO species.

MPCAs AND DETECTION METHODS

The model MPCAs utilized in these studies provided a range of fates and persistences in the enclosed multi-species test system. In the two trials (1 and 5) in which B. sphaericus spores were used as the model MPCA, the organism persisted in saltwater throughout the 28 day duration of the two studies. This was not unanticipated because B. sphaericus spores are known to remain viable in soil for considerable periods of time (Hertlein et al., 1979) and to remain visibly unaffected during passage through the gut of mosquitoes (Davidson, 1979; 1981).

The detection method used to track B. sphaericus in Trials 1 and 5 was simple and easy to use. The organism's presence or absence could be readily detected and accurately enumerated. Histological studies of NTOs in Trials 1 and 5 showed the presence of large numbers of Gram positive bacilli and spores in the gut contents of some of the NTOs from the exposed tanks. This observation suggests that the model MPCA may have cycled through the food chain. However, although this model MPCA did persist for at least 30 days in the test system while losing three logs activity, it did not cause observable pathological

anomalies in the NTOs used in this study (Tables 13, 14, 15 and 18).

In marked contrast to the findings when bacillus spores were used as the model MPCA, the vegetative cells of the strain of B. sphaericus used in Trial 2 became undetectable in the seawater system within the first 24 hr (Table 16). Histological study of the NTOs in this trial also suggested that the NTOs consumed the MPCA, but that its presence caused no pathological anomalies (Table 15). Davidson (1979; 1981) noted that B. sphaericus vegetative cells are digested very rapidly after entering the anterior midgut of mosquito larvae. Broken cell walls were detected within 30 min of ingestion, and defecation removed nearly all the bacteria from the mosquito larva's gut within 1 hr. Spores may remain visibly unaffected by digestion. Thus, the Gram positive bacilli noted in the gut contents of some of the NTOs in our studies may be cells which were in the gut less than 30 min before we sampled and preserved the specimens in Davidson's fixative.

Brownbridge and Margalit (1987) noted that many factors contribute to the disappearance and inactivation of such bacterial strains in the environment. Our inability in Trial 2 to recover viable B. sphaericus vegetative cells after 24 hr from our test system suggests that the bacterial cells were destroyed by environmental effects and possibly by the NTOs. This route of MPCA clearence from the test tanks is a possibility because one large oyster may filter nearly 400 L of seawater in 24 hr (Bailey and Biggs, 1968). As each 120 L tank contained 15 oysters at the start of each trial, the entire volume of tank water may have passed through the oysters as many as 50 times in the first 24 hr. If only a fraction of the viable B. sphaericus cells were inactivated during each passage through the gut of an oyster, it is possible that the entire dose of MPCA could be reduced to zero in a single day.

As was the case with the detection method used for B. sphaericus in Trials 1 and 5, the detection method for this strain of the MPCA was also simple. Both of these Bacillus detection methods consisted of using standard microbiological culturing techniques to detect viable organisms with unique genetic markers.

The effects of the B. sphaericus toxin are not well understood at the molecular and cellular levels. Kellen et al. (1965) noted that signs of toxicity in susceptible larvae when fed less insecticidal strains of B. sphaericus may not occur for three days, but then posterior midgut cells demonstrated vacuolation and sloughing. Other toxin-mediated bacterial and fungal infections of insects may produce similar changes in midgut cells (Ebersold et al., 1977; Zacharuk, 1971). Digestion of B. sphaericus cells may release the toxin, possibly causing neurotoxicity (Davidson, 1981). Rapid swelling of the midgut of larval mosquitos resulting from an influx of fluid into the gut lumen is the first visible symptom of intoxication in susceptible

larvae. The gut contents within the peritrophic membrane display zigzag folds as swelling progresses until the midgut wall is displaced against the outer body wall, eliminating most of the hemocoelic space. Ten hours after feeding B. sphaericus to susceptible mosquito larvae, cytolysosomes in the midgut become larger and more numerous. Midgut cells swell and separate from each other at the bases. Loss of microvilli from anterior midgut cells and cytolysis and sloughing of posterior midgut cells occurs, with larvae becoming moribund or dead by 24 hr (Davidson, 1979). The classic signs and lesions of B. sphaericus toxicity, described by Davidson (1979; 1981), were not noted during histological examination of paraffin sections of exposed NTOs in this study.

Pseudomonas putida used as a model MPCA in Trials 4 and 6 showed variable results. In the saltwater test system, it did not survive more than 5 days (Table 17), but in the freshwater system it survived and was detectable in some samples assayed for the 29-day study (Table 19). This is not surprising since Pseudomonas spp. are naturally found in freshwater and can even persist in distilled water (Doudoroff and Palleroni, 1974). The microbiological culturing method was excellent for tracking this organism in the test system and in the tissues of the NTOs. The combination of the two antibiotic resistance genes, in addition to the biochemical properties inherent in this Pseudomonas sp. (i.e., turning Ps F Agar yellow under its colonies), simplified isolation, identification, and enumeration of this genetically engineered microorganism.

The gene probe assay for tracking this organism (Pseudomonas) in saltwater (Trial 4) also worked very well. The positive results were indicated by strong signals and there was not any background nor any non-specific binding of the probe. However, in the freshwater system (Trial 6), this assay was not sufficient. Something appeared to cause non-specific binding or cross reaction. It is easy to imagine that in the freshwater system that one or more other Pseudomonas sp. existed which caused interference with the assay. If a gene probe method for this organism is to be used in the future in a freshwater system, investigations will be necessary with the gene probe and determine the extent of the interference.

The results of Trials 3 and 7, in which the baculovirus AcMNPV was used, are difficult to assess. In these trials we employed a gene probe in an attempt to detect and track the fate and persistence of the viral DNA of this model MPCA. However, this method was not as simple and easy as the microbiological culturing methods used with the bacterial model MPCAs. Hence, in our hands, it was not possible to ascertain whether or not the MPCA persisted in either the seawater or freshwater test systems, since the initial background studies indicated that the gene probe did not produce strong signals (and therefore not a strong affinity) to target viral DNA (of virus in the occlusion bodies). The sequence itself contained in the insert may not have a strong enough affinity for its target sequence. If this MPCA is used in

future studies, it will be necessary to investigate more fully a gene probe to this MPCA.

Further studies should include attempts to analyze water and tissue homogenates for specific antigen or nucleic acid from the model MPCAs using either monoclonal antibodies or gene probes. It is of interest to study the fate of persistence of not only the intact viable model MPCA itself, but its genetic material. Use of the polymerase chain reaction to amplify the genetic material (Steffan and Atlas, 1988) may enhance sensitivity of detection when using gene probes.

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